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Anaeroplasma bactoclasticum extracellular amylase

by

Christina Jost

A Dissertation Submitted to the

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INTRODUCTION

There have been numerous studies of amylolytic enzymes, especially over the past few decades. A primary reason for these studies was the great industrial importance of these enzymes. Amylases have wide industrial applications, including starch size removal from textiles, preparation of adhesives, development of sizes and coatings for the paper industry, manufacture of glucose and syrup, and the degradation of barley adjuncts in brewing and other fermentation processes (Burbidge and Collier, 1968).

The study of amylases also has important agricultural applications. The maximum utilization of starch in feed grains by ruminants, for example, is dependent primarily on the amylases produced by the microbial flora of the rumen. A greater knowledge of these amylases and their action would result in a better understanding of ruminant nutrition. However, only three amylases from rumen microorganisms have been characterized. These are Streptococcus bovis (Hobson and MacPherson, 1952; Walker, 1965; Walker and Hope, 1964), Clostridium butyricum (Hobson and MacPherson, 1952; Walker and Hope, 1964) and Bacteroides amylophilus (McWethy and Hartman, 1977).

The object of this dissertation was to examine the production, isolation and characterization of an amylase(s)

produced by an obligately anaerobic rumen mycoplasma, Anaeroplasma bactoclasticum. It was believed that this unique organism might produce an amylase(s) with unusual properties. I did, in fact, discover that A. bactoclasticum produced amylase(s) that differed from known amylases in behavior during purification and in action pattern.

LITERATURE REVIEW

The Mycoplasmas

The mycoplasmas are the smallest known procaryotes. Unlike other procaryotes they have no cell wall (Razin, 1978). They do contain the minimum constituents necessary for cell growth and replication. Freundt (1974) describes two genera of the class Mollicutes. The genus Mycoplasma requires sterols for growth, while the genus Acholeplasma does not require them (Freundt, 1974; Maniloff and Morowitz, 1972). Recently, other genera have been established. Among these is the genus Ureaplasma, or T-mycoplasmas, which require urea for growth (Razin, 1978); the genus Spiroplasma, members of which resemble spirochetes because they form helical motile filaments (Davis et al., 1972); and the genus Thermoplasma, members of which have optimum growth temperatures of about 59 C (Belly, Bohlool and Brock, 1973). Mycoplasmas have been isolated from animals, insects and plants (Razin, 1978). Studies utilizing the electron microscope suggest that cell wall-less procaryotes, possibly mycoplasmas, are present in amphibians (Babudieri, 1972), mollusks (Harshbarger, Chang and Otto, 1977), trematodes (Morris and Halton, 1975) and fungi (Heath and Unestam, 1974; Ross, Pommerville and Damm, 1976). It is generally believed that many mycoplasmas exist that have not yet been isolated.

Anaerobic Mycoplasmas

In 1966 Hungate described a procaryote isolated from the contents of the bovine rumen. This organism, originally described as a proteolytic, nonsporeforming bacterium (Hungate, 1966), was later identified as an obligately anaerobic mycoplasma (Robinson and Hungate, 1973). These organisms were found in numbers ranging from 10^4 to 10^7 colony-forming units per gram of ruminal contents in the original study. Hungate suggested that they may live on other rumen bacteria because they possessed a bacterioclastic enzyme. This conclusion was based on the observation (Hungate, 1966) that the numbers of these mycoplasmas increased when the substrates for other rumen organisms were depleted.

Hungate's organism was subsequently characterized by Robinson and Hungate (1973) as a mycoplasma. The organism was classified as a mycoplasma on the basis of colonial morphology, microscopic morphology, filterability through a 0.45-μm membrane and resistance to penicillin G (Robinson and Hungate, 1973). It is unlikely that this mycoplasma was an L-form of a rumen bacterium, since no reversion to a walled morphology occurred when the microorganism was isolated and subcultured in the absence of cell wall inhibitors (Robinson and Hungate, 1973). The organism was unique in that it possessed an extracellular caseinase and was

bacteriolytic. This strain was Gram negative, usually coccoid, and possessed a unit membrane structure. Growth was dependent upon strict anaerobic conditions. Arabinose, ribose, xylose, galactose, glucose, lactose, maltose and sucrose were fermented. When galactose was fermented, formate, acetate, lactate, carbon dioxide and hydrogen were produced.

J. P. Robinson and R. E. Hungate (1973) could not demonstrate a sterol requirement for this organism. Consequently, they assigned the organism to the genus Acholeplasma. The organism was physiologically different from other species of Acholeplasma, so the new species, Acholeplasma bactoclasticum, was established.

Subsequent research by I. M. Robinson and M. J. Allison (1975) showed that Robinson and Hungate's strain (strain JR) required cholesterol for growth. The cholesterol requirement had been previously overlooked because enough cholesterol for this strain's growth was either supplied by killed Escherichia coli cells, present in the medium to detect the bacterioclastic enzyme, or as a trace contaminant in the trypticase that was a component of the culture medium. Experimentation using cholesterol-free media, lacking killed E. coli and containing trypticase that was extracted with ethyl ether, showed conclusively that strain

JR was dependent on cholesterol for growth. Sensitivity to digitonin also confirmed that sterols were required for growth (Robinson and Allison, 1975). Digitonin lyses mycoplasmas by forming a complex with membrane cholesterol (Bernheimer and Davidson, 1965). Since Acholeplasma was not the proper genus for this mycoplasma, they proposed the formulation of the genus Anaeroplasma, based on its unique requirement of obligate anaerobic conditions for growth (Robinson and Allison, 1975). All other known mycoplasmas which grow anaerobically can also grow aerobically (Vandemark, 1969). After obtaining several isolates of this mycoplasma from the rumens of cattle and sheep (Robinson and Allison, 1975; Robinson, Allison and Hartman, 1975), it was evident that there were strains that required cholesterol for growth and those that were not cholesterol-dependent. Thus, cholesterol dependence was not a good criterion for genus specification. This is significant because the present classification schema for mycoplasmas (Freundt, 1974) was based on sterol requirements. The presence of sterol-dependent and sterol-independent strains in the genus Anaeroplasma indicate an examination of the Mycoplasmataceae classification schema is necessary.

Habitat of *Anaeroplasma*

The habitat of *Anaeroplasma* appears to be limited to the bovine and ovine rumen. Robinson and Hungate (1973) reported that bacteriolytic strains were found in both cattle and sheep rumens, where the viable count was not greater than 10^7 *Anaeroplasma* per ml rumen contents. They concluded that it was unlikely that such high numbers in the rumen were due to organisms entering the animal from the nares or buccal cavity. This hypothesis was reinforced by evidence that the animal's feed and water contained very low levels of the organism. The strict anaerobic character of these mycoplasmas also indicated that the rumen was the natural habitat of *Anaeroplasma*.

Anaerobic mycoplasmas have been isolated from ruminants from several geographic locations and fed on a variety of diets (Robinson, 1979). *Anaeroplasma* apparently is ubiquitous in the rumen of sheep and cattle (Robinson, 1979). *Anaeroplasma* has not been isolated from human feces (Robinson and Hungate, 1973), the deer rumen (Robinson, Allison and Hartman, 1975; Robinson and Hungate, 1973), or fecal material from rabbits, hamsters, horses, pigs or turkeys (Robinson, Allison and Hartman, 1975).

J. P. Robinson and R. E. Hungate (1973) were the first to isolate bacteriolytic mycoplasma from the rumen. By

using similar methods, I. M. Robinson, Allison and Hartman (1975) discovered that nonbacteriolytic, anaerobic mycoplasma also were present in rumen contents. The lytic mycoplasmas were present at between 10^5 and 10^7 colony-forming units per gram (cfu/g) of rumen contents; the nonlytic mycoplasmas were present in numbers of 10^7 to 10^8 cfu/g of rumen contents (Robinson, Allison and Hartman, 1975). Examination of the rumen contents from several animals showed that the numbers of nonlytic mycoplasmas were consistently higher than those of lytic mycoplasmas (Robinson, 1979).

The ecological role of these rumen mycoplasmas has not been determined (Robinson, 1979). The numbers of anaerobic mycoplasmas (10^5 to 10^7 cfu/g) were low, compared to numbers of other rumen microorganisms (approximately 10^{10} cfu/g). It was believed, however, that these mycoplasmas contributed to the pool of fermentation products in the rumen. Also, the bacteriolytic species may contribute significantly to the high microbial turnover rate in the rumen (Robinson, 1979). This turnover rate has been shown to be as high as 20% (Robinson, 1979).

Characteristics of *Anaeroplasma*

Within the genus *Anaeroplasma* two species have been established (Robinson, 1973; Robinson, Allison and Hartman, 1975). These are *A. bactoclasticum*, which has bacteriolytic properties, and *A. abactoclasticum*, which is not bacteriolytic. *A. abactoclasticum* is the type species of the genus. The type strain of this species (strain 6-1, ATCC 27879) is nonbacteriolytic and sterol-requiring (Robinson, 1979). The type strain of *A. bactoclasticum* is strain JR, the original isolate of Robinson and Hungate (Robinson, 1979).

Examination of a number of strains indicated that the genus *Anaeroplasma* was composed of obligately anaerobic mycoplasmas that were usually coccoid, approximately 500 nm in diameter, Gram negative, and nonmotile (Robinson, Allison and Hartman, 1975). Phase contrast microscopy revealed a microscopic morphology that included single cells, clumps, dumbbell forms and clusters of 2 to 10 cocci joined by short filaments. Negatively stained preparations observed by transmission electron microscopy showed a variety of pleomorphic forms. Among these were budding, bleb-like structures and filamentous forms. Electron microscopy of thin sections revealed that no cell walls were present; each cell was bound only by a trilaminar membrane.

The colonial morphology was similar to that of "classical mycoplasmas", since surface colonies had a transparent periphery and a dense center, or "fried-egg" appearance. Surface colonies had an average diameter of 1 mm. Subsurface colonies were golden and irregularly shaped. The colonial appearances of lytic and nonlytic Anaeroplasma were identical (Robinson, Allison and Hartman, 1975).

Several Anaeroplasma strains have been assayed for fermentation products by gas chromatography (Robinson and Allison, 1975; Robinson, Allison and Hartman, 1975; Robinson and Hungate, 1973). Ethanol, acetate and lactate were produced by all of the strains tested. Strain JR fermented galactose to acetate, formate, lactate, propionate, carbon dioxide, hydrogen and ethanol (Robinson and Hungate, 1973). Strain 6-1 fermented uniformly ^{14}C-labeled starch to 36.6% acetate, 19.0% formate, 17.2% lactate, 8.2% carbon dioxide, 4.8% ethanol, 1.1% succinate and 5.6% of several unidentified products (Robinson, Allison and Hartman, 1975). Some Anaeroplasma strains other than strain JR also produced hydrogen (Robinson, 1979).

Among the mycoplasmas, deoxyribonucleic acid (DNA) comprises about 4 to 7% of the cell dry weight (Maniloff and Morowitz, 1972). The DNA base ratios of all the known mycoplasmas lie in the range of 23 to 41 mol% guanine plus

cytosine (G+C) (Maniloff and Morowitz, 1972). More specifically, the base ratio ranges were: Acholeplasma, 30-36 mol%; Mycoplasma, except M. pneumoniae, 23-36 mol%; M. pneumoniae 39-41 mol%, and Ureaplasma, 28 mol% (Maniloff and Morowitz, 1972). Studies of the G+C base composition of Anaeroplasma DNA revealed different base ratios for sterol-requiring strains and those that did not require sterols (Robinson, Allison and Hartman, 1975). Cholesterol-dependent strains had base ratios of 29 mol%, while 40 mol% was the ratio found in strains not requiring cholesterol for growth (Robinson, 1979; Robinson, Allison and Hartman, 1975).

The obligate anaerobic nature of Anaeroplasma is unique among the mycoplasmas. The presence of plasmalogens (alk-1'-enyl glyceryl ethers) have been demonstrated in a number of species of anaerobic bacteria (Goldfine and Hagen, 1972). Thus, finding that plasmologen was a major component of the polar lipids of Anaeroplasma supported the contention that these organisms were distinct from aerobic mycoplasmas (Langworthy, Mayberry and Robinson, 1975). The lipid composition of the Anaeroplasma strains was similar to the composition of aerobic mycoplasmas, except for the presence of plasmalogen (Langworthy, Mayberry and Robinson, 1975). Plasmalogen was present in both bacteriolastic and nonlytic strains of this mycoplasma (Langworthy,

Mayberry and Robinson, 1973).

Serology has been so important in distinguishing classical mycoplasmas that Freundt defined a mycoplasma species as "a group of strains so closely related that an antibody produced against one strain will prevent replication and metabolism in all other strains within that group" (Razin, 1978). Consequently, an investigation of the serological typing of Anaeroplasma strains was of great interest. Four serovars of Anaeroplasma were identified by Robinson and Rhodes (1977) when experimenting with ten strains from cattle and sheep. The four serovars corresponded with grouping based on cultural, biochemical and biophysical properties. Within each serovar, strains showed cross-agglutination, but there was no cross-agglutination among the four serovars. No species cross reactions were observed when tested with antigens of known aerobic bovine Mycoplasma and Acholeplasma strains (Robinson and Hungate, 1973; Robinson and Rhodes, 1977). These four groups of Anaeroplasma strains were confirmed by growth inhibition, agglutination and gel diffusion tests (Robinson and Rhodes, 1977). The grouping of the Anaeroplasma strains was similar when based on either serological or biochemical data, except for strain JR. This strain resembled other lytic mycoplasmas biochemically but belonged to a different serovar (Robinson and Rhodes, 1977).

Since only a limited number of strains were examined, further testing of more isolates should reveal other serological and physiological groups. These data are important because they confirm the lack of relationship with other aerobic mycoplasmas, justifying the creation of the genus Anaeroplasma.

Anaeroplasma Nutrition Studies

Studies of the nutritional requirements of classical aerobic mycoplasmas have been reviewed (Razin, 1969; Razin, 1973). It is generally believed that nutritionally inadequate artificial media has prevented the cultivation of other mycoplasmas in nature (Razin, 1969; Simmons and Lukert, 1972). It has also been noted that the formation of the typical mycoplasma "fried-egg" colonial morphology was dependent on the nutritional and physical properties of the medium (Razin, 1969). Consequently, there has been some study of the nutritional requirements of Anaeroplasma.

Robinson and Hungate (1973) and Robinson and Allison (1975) used media based on formulations previously used for rumen bacteria (Bryant and Burkey, 1953). Anaerobic methods were used to establish conditions similar to those found in the rumen, particularly the highly reduced conditions. Obligate anaerobic conditions were necessary

for growth; Anaeroplasma would not grow on agar plates of clarified rumen medium containing cysteine or sodium sulfide as reducing agents when incubated aerobically or in a reduced oxygen atmosphere in Brewer jars (Robinson and Hungate, 1973). Growth was inhibited by oxygen. Thus, if the oxidation-reduction indicator, resazurin, was oxidized (approximately -51 mV; Holdeman, Cato and Moore, 1977), Anaeroplasma would not grow (Robinson, Allison and Hartman, 1975). Clarified rumen fluid was added to the media to simulate the natural environment of these anaerobic mycoplasmas (Robinson and Allison, 1975; Robinson, Allison and Hartman, 1975; Robinson and Hungate, 1973). The osmotic pressure of bovine rumen fluid is about 7.8 atmospheres (Hungate, 1966). This is within the range of 6.8 to 14.0 atmospheres which is optimal for most mycoplasmas (Leach, 1962). The estimated internal osmotic pressure of Acholeplasma laidlawii is 5 to 6 atmospheres (Spears and Provost, 1967). Therefore, it was not surprising that mycoplasmas remained stable in an environment containing rumen fluid. I. M. Robinson (1973) reported that 40% clarified rumen fluid was the optimum level for growth of Anaeroplasma. All strains of Anaeroplasma grew in a mineral salts medium containing soluble starch and clarified rumen fluid (Robinson, Allison and Hartman, 1975). Anaeroplasma would not grow in

clarified rumen fluid without an added carbon source (Robinson, 1979).

Besides media containing rumen fluid, Robinson, Allison and Hartman (1975) were able to grow the organism in a medium not containing rumen fluid, a partially defined modification of medium-10 (Caldwell and Bryant, 1966). Testing carbon sources showed that growth was optimal with soluble starch (Robinson, Allison and Hartman, 1975). Lower numbers were obtained when maltose, cellobiose or glucose were used as carbon sources. Arabinose, fructose, galactose, glycerol, mannose, raffinose, salicin, sucrose, or xylose did not support growth. None of the strains grew in modified medium-10 (MM-10) without a carbon source whether or not cholesterol was added (Robinson, Allison and Hartman, 1975).

Cholesterol has been shown to be essential for growth for some strains of Anaeroplasma, but not for others (Robinson, Allison and Hartman, 1975). The amount of cholesterol required for growth varied with the strain (Robinson, Allison and Hartman, 1975). It has been reported that rumen fluid contains approximately 1.7 µg of cholesterol per ml (Robinson, 1972). Robinson (1979) found that the optimum cholesterol concentration was 10 µg per ml, while 100 µg per ml was slightly inhibitory. Increased growth of cholesterol-independent strains was reported when cholesterol was added to the medium (Robinson, 1979).

Research on aerobic mycoplasmas indicated that rather than just being adsorbed on the membrane surface from the growth medium, cholesterol was incorporated as part of the mycoplasma membrane (Razin, 1969; Razin, 1978). Cholesterol appeared to be incorporated into membranes by a nonenergy requiring process (Smith and Rothblat, 1960). When both esterified and free cholesterol were present in the growth medium, mycoplasmas selectively incorporated the free cholesterol (Argaman and Razin, 1965).

Anaeroplasma have been grown in chemically defined media (Robinson, 1979). All strains tested have required an exogenous source of phospholipid (Robinson, 1979). Increased growth was observed when media were supplemented with 0.01 to 1.0 mg/ml of lipopolysaccharide (LPS). When LPS was hydrolyzed at pH 10 for 60 min at 100 C, no growth stimulus was observed. Thus, it appeared that a factor present in LPS was essential for growth. This growth factor was extracted into chloroform-ethanol after mild acid hydrolysis (Robinson, 1979). Also, the LPS could be replaced by phosphatidyl choline, and the phosphatidyl choline also lost its growth-stimulating properties after hydrolysis at pH 10 for 60 min at 100 C. Phosphatidyl glycerol, diphosphatidyl glycerol, lysophatidyl choline and phosphatidic acids all supported growth but not to the same degree as LPS. Lipids that would not replace LPS included glycerol phosphoryl

serine and glycerol phosphate (Robinson, 1979). Other substances tested included monoolein, diolein, triolein, lauric, myristic, myrisoleic, palmitic, palmitoleic, stearic, oleic, linoleic, linolenic, arachidic, cis-11-eicosenic and archidonic acids. None of these, either alone or combined with glycerol or choline, would replace phospholipids (Robinson, 1979). Thus, the requirement for this growth factor can be fulfilled by lipopolysaccharide or phosphatidyl choline (Robinson, 1979).

There has also been some study on other growth factors; however, requirements for minerals, vitamins, and nitrogen have not been defined. Studies with strain 6-1 indicated that better growth was obtained when trypticase was added to the medium than when an amino acid mixture was used (Robinson, 1979). Like most rumen bacteria, Anaeroplasma do not require nucleic acids for growth (Robinson, 1979). This characteristic is unlike most mycoplasmas which require nucleic acids for growth. Acetate also was required for growth; this result was unexpected because a relatively insignificant amount of ^{14}C-labeled acetate was actually incorporated during growth. These results imply that Anaeroplasma cannot synthesize long-chain fatty acids from acetate (Robinson, 1979).

Studies of the effects of various potential inhibitors on

Anaeroplasma showed that growth was inhibited by bacitracin (0.7 µmol/ml), streptomycin (0.36 µmol/ml), thallous acetate (0.75 µmol/ml)(Robinson, Allison and Hartman, 1975) and tetracycline (20 µg/ml) (Robinson and Hungate, 1973). Robinson, Allison and Hartman (1975) found that Anaeroplasma was not inhibited by 1000 U/ml of penicillin G, and Robinson and Hungate (1973) reported that strain JR was not inhibited by 5000 U/ml of penicillin G. The lack of sensitivity to this cell wall inhibitor confirms the electron microscopic observations of this organism.

Enzymes of Mycoplasmas

Smith (1971) has reviewed the subject of mycoplasma exoenzymes. By comparison to other microorganisms very few enzymes from mycoplasmas have been studied. A few enzymes are associated with the mycoplasma membrane. Among these enzymes are a reduced nicotinamide adenine dinucleotide oxidase (Pollack, Razin and Cleverdon, 1965), cholesterol esterase (Smith, 1959), a glucosyl diglyceride synthetase (Smith, 1969) and a phosphatidyl glucose synthetase (Smith, 1971). All of these enzymes were produced by A. laidlawii. Other membrane-associated enzymes include a cholesteryl glucoside synthetase of M. gallinarium, the quinone and ferricyanide reductases of M. gallisepticum (Smith, 1971) and adenosine triphosphatase, ribonuclease,

and deoxyribonuclease of several Mycoplasma species (Pollack, Razin and Cleverdon, 1965).

There have also been some studies of the enzymes of glycolysis (Razin, 1969), primarily in M. mycoides (Rodwell, 1960; Rodwell, 1967), M. gallisepticum (Tourtellotte and Jacobs, 1960) and A. laidlawii (Castrejon-Diez, Fischer and Fischer, 1963). Also, glucosidases have been found in A. laidlawii and M. gallinarum, but not in M. arthritidis (Henrikson and Smith, 1964). The latter authors found that A. laidlawii strain B and M. gallinarum possessed β-glucosidases, while A. laidlawii possessed α-glucosidases. The β-glucosidase of A. laidlawii strain B was partially characterized. Another study of A. laidlawii (Migushina et al., 1974) indicated that a specific maltose transport system was present in conjunction with an intracellular maltase. A study of M. arthiritidis revealed the presence of a proteolytic enzyme that attacked denatured collagen; unlike other proteolytic enzymes, it would not attack α-casein. Some exoenzymes, including nucleases and enzymes that alter erythrocyte antigenic determinants, have been detected, and these may be partially responsible for the effects of mycoplasmas on animal cells (Maniloff and Morowitz, 1972; Stanbridge, 1971). The production by mycoplasmas of other extracellular proteins, such as hemolysins and neurotoxins, has been reviewed (Smith, 1971).

Anaeroplasma was originally studied because of interest in its extracellular bacteriolytic enzyme (Hungate, 1966). Robinson and Hungate (1973) observed zones of clearing around Anaeroplasma bactoclasticum strain JR colonies growing in agar containing killed Escherichia coli. An ammonium sulfate precipitate of this enzyme lysed the peptidoglycan layer of autoclaved rumen bacteria, E. coli, Salmonella typhimurium, and Spirillum serpens. Killed cells of Micrococcus lysodeikticus cells, however, were not lysed. Most strains of Anaeroplasma have been observed to lyse only autoclaved cells; however, some strains have been reported that lysed both autoclaved and living Gram negative bacteria (Robinson, 1979).

Anaeroplasma strains that possess an extracellular protease have also been observed (Robinson, 1979; Robinson and Hungate, 1973). This enzyme hydrolyzes casein, but ammonia is not produced. Robinson and Hungate (1973) hypothesized that both the proteolytic and the bacteriolytic activities were properties of the same enzyme. Their hypothesis was based on the following observations: (i) both proteolysis and cell lysis were reversibly inhibited by oxidation, (ii) both proteolytic and lytic activities could be restored by the reducing agents mercaptoethanol and dithiothreitol, (iii) ammonium sulfate precipitation increased both enzyme activities to the same degree, and

(iv) both proteolytic and lytic activities were eluted as a single peak from a Sephadex G-100 column. Furthermore, both activities had the same pH and temperature optima. Besides the bacteriolytic enzyme, I. M. Robinson (personal communication, National Animal Disease Laboratory, Ames, Iowa, 1975) observed that Anaeroplasma bactoclasticum strain 5LA possessed an extracellular amylase. The study of this amylase is the topic of this dissertation.

General Properties of Amylases

Amylases are hydrolytic enzymes that catalyze the cleavage of the α1:4 glucosidic linkage in polysaccharides. Carbohydrates such as starch, glycogen, amylose and amylopectin are hydrolyzed by these enzymes. There are basically two types of amylase: α-amylases and β-amylases. The α-amylases are endoenzymes that produce a variety of low molecular weight maltodextrins with the α-configuration at carbon one of the reducing glucose unit. These enzymes have the ability to bypass α1:6 branch points. The α-amylases have been isolated from various plants, animals and microorganisms (Robyt and Whelan, 1968). The β-amylases are exoenzymes which produce β-maltose and do not possess the ability to bypass α1:6 branch points (Robyt and Whelan, 1968). The β-amylases have been isolated exclusively from

plants (Robyt and Whelan, 1968), with the exception of Bacillus polymyxa amylase which, unlike other α-amylases, produced β-maltose as the main hydrolytic product. This enzyme was similar to the α-amylases because it could bypass α1:6 branch points (Robyt and French, 1964). Another group of enzymes similar to the amylases is the glucoamylases (Fleming, 1968). These exoenzymes catalyze the hydrolysis of starch; β-glucose is liberated from the nonreducing end. Reportedly, some glucoamylases also hydrolyze α1:6 branch points; however, this phenomenon may not be characteristic of glucoamylases, but rather an artifact caused by the use of impure enzyme preparations (Fleming, 1968). Glucoamylases have been isolated primarily from molds and animal tissue (Fleming, 1968).

Some physical properties of certain microbial amylases are shown in Table 1. In general, the α-amylases have a molecular weight of approximately 50,000, a pH range of 4.5 to 7.0 with a pH optimum near 6.0, and a temperature optimum near 40 C (Robyt and Whelan, 1968). There are, however, striking exceptions to these parameters (Boyer, Ingle and Mercer, 1973; Ingle and Boyer, 1976). Information concerning α-amylase amino acid composition and catalytic groups has been reviewed (Fischer and Stein, 1960; Manners, 1962; Pazur, 1965; Robyt and Whelan, 1968; Takagi,

Table 1. Some physical properties of several microbial amylases

Organism	pH		Temperature		MW	Reference
	Optimum	Range	Optimum	Stability		
Bacillus amyloliquefaciens	5.9	5.5-6.5	65 C	-	-	Walker and Campbell, 1967
Bacillus amyloliquefaciens SB, T and F	5.9	5.5-6.5	45 & 65 C	-	-	Walker and Campbell, 1967
Bacillus caldolyticus	-	-	70 C	90 C	-	Grootegood, Lauwers and Heinen, 1973
Bacillus stearothermophilus	5.0	-	65 C	-	52,700	Manning and Campbell, 1961
Bacillus stearothermophilus	5.0	4.5-6.5	65-73 C	-	48,000	Ogasahara, Imanishi and Isemura, 1970
Bacillus subtilis SAC, MAR, NA64	6.8	5-9	-	68 C	55,000	Matsuzaki et al., 1974
Bacillus subtilis NA20	6.1	5-9	-	57 C	42,000	Matsuzaki et al., 1974
Bacillus subtilis NAT	6.1	5-9	-	57 C	34,000	Matsuzaki et al., 1974
Bacillus subtilis LIQ	6.1	5-9	-	80 C	-	Matsuzaki et al., 1974
Bacillus subtilis W23	6.3	5.7-6.7	65 C	-	-	Welker and Campbell, 1967
Bacillus subtilis	-	-	-	-	96,000 tetramer	Robyt and Ackerman, 1973
Bacteroides amylophilus	6.3	5.8-7.5	43 C	-	92,000	McWethy and Hartman, 1977
Clostridium butyricum	5.4-6.0	-	48 C	-	-	Hobson and MacPherson, 1952 Walker and Hope, 1964
Enterobacter aerogenes	6.8	5.5-8.0	50 C	-	54,000	Kainuma et al., 1975
Halobacterium halobium	6.4-6.6	-	55 C	-	-	Good and Hartman, 1970

Table 1 (Continued)

Organism	pH		Temperature		MW	Reference
	Optimum	Range	Optimum	Stability		
Streptococcus bovis	5.8	-	50 C	-	-	Walker and Hope, 1964
Streptococcus equinus	7.0	-	38 C	-	-	Boyer and Hartman, 1971

Toda and Isemura, 1971; Thoma, Dygert and Spradlin, 1971).

Divalent cations have been reported to be significant in maintaining the activity and stability of some microbial extracellular enzymes (Vallee et al., 1959). The effect of calcium on the stability of α-amylases was first reported by Wallerstein in 1909; who patented the use of calcium in the brewing process (Vallee et al., 1959). Since then, calcium has been shown to be important for the stability and activity of many extracellular amylases (Boyer and Hartman, 1971; Fischer and Stein, 1960; Fogarty and Griffin, 1973; Levitzki and Reuben, 1973; Manning and Campbell, 1961; Ogasahara, Imanishi and Isemura, 1970; Yamane and Maruo, 1974).

Calcium was first identified as an amylase cofactor when it was shown that calcium could reactivate previously sequestered amylase (Vallee et al., 1959). Several authors have observed that treatment with the chelating agent ethylene diamine tetraacetic acid (EDTA) inactivated α-amylase (Fischer and Stein, 1960; Stein and Fischer, 1959; Vallee et al., 1959). It is generally believed that the only effect of EDTA on α-amylases is binding metal ions and that all other conformational changes are a result of chelation (Stein and Fischer, 1959). Yamamoto (1956) first noted the preventative effect of starch on amylase inhibition by EDTA. Vallee et al. (1959) proposed that this indicated either the forma-

tion of an enzyme-substrate complex tightened the amylase configuration and strengthened the binding of calcium, or the EDTA interacted with the amylase at the calcium ion. Since studies have indicated that calcium does not take part in catalysis (Hsiu, Fischer and Stein, 1964), the former hypothesis is indicated.

When calcium ions were added to a preparation previously treated with sequestering agents, enzyme activity was restored (Grootegood, Lauwers and Heinen, 1973; Stein and Fischer, 1959; Taha, Mahmoud and Attia, 1968; Vallee et al., 1959). Calcium functioned by giving stability to the enzyme configuration. This cation also protected amylases from proteolysis and heat denaturation (Fischer and Stein, 1960; Greenwood and Milne, 1968; Hagihara et al., 1956; Isono, 1970; Kainuma et al., 1975; Manning and Campbell, 1961; Markovitz, Klein and Fischer, 1956; Stein and Fischer, 1959). Consequently, when calcium was removed amylases were more susceptible to proteolysis (Stein and Fischer, 1959; Vallee et al., 1959; Yamamoto, 1956). Stein and Fischer (1959) showed that calcium not only retarded the proteolysis of amylase, but when calcium ions were added to amylase undergoing proteolytic degradation the amylase breakdown was greatly reduced. Resistance of amylase to proteolytic enzymes is important because most bacterial amylases are formed in the presence of high protease concentrations (Stein and

Fischer, 1959).

In several studies the amount of calcium associated with the enzyme has been calculated. Thus, it has been shown that most amylases contain a minimum of one gram-atom per mole of enzyme (Manning and Campbell, 1961; Ogasahara, Imanishi and Isemura, 1970; Stein and Fischer, 1959, Vallee et al., 1959).

Several ions, besides calcium, have been studied with respect to their effects on amylases. Amylases of mammalian origin are activated by monovalent anions (Greenwood and Milne, 1968). Chloride ions, for example, are important for salivary amylase activity (Walker and Hope, 1963). Some microbial amylases also have increased activity when chloride ions are present; among these are the amylases of Halobacterium halobium (Good and Hartman, 1970) and Streptococcus equinus (Boyer and Hartman, 1971). Zinc ions have been shown to be associated with some Bacillus amylases. B. subtilis α-amylase contains both calcium and zinc. The zinc functions as a center of dimerization (Vallee et al., 1959). Robyt and Ackerman (1973) reported a 96,000 dalton zinc tetramer of B. subtilis α-amylase. Fischer and Stein (1960) also observed zinc dimerization in B. amyloliquefaciens α-amylase.

Smolka, Birnbaum and Darnall (1971) showed that the rare earth metals could substitute for calcium in B. subtilis

α-amylase. Although Levitzki and Reuben (1973), using different techniques, were unable to obtain activation of this amylase with lanthanide ions, Darnall and Birnbaum (1973) confirmed that lanthanide metals activated a B. subtilis α-amylase.

Kato et al. (1975) studied the effect of 17 metal ions on Pseudomonas amylase activity. Activity was completely inhibited by mercury, copper and silver, while iron, lead and cadmium reduced the activity to 49%, 53% and 65%, respectively. Stein and Fischer (1959) reported that divalent cations, including magnesium, calcium, barium, manganese and nickel, decreased amylase susceptibility to protease. However, the cations did not all protect the amylase to the same degree. The activity of Enterobacter aerogenes amylase was increased by calcium and strontium ions strongly inhibited by cupric and zinc ions, and completely inhibited by mercuric ions (Kainuma et al., 1975). Mahmoud, Taha and Attia (1968) found that a combination of iron (II), magnesium and zinc restored α-amylase activity following chelation, while various combinations of any two of these three ions did not restore activity. Thus, metals that inhibit α-amylases include mercury (Di Carlo and Redfern, 1947; Greenwood and MacGregor, 1965; Greenwood, MacGregor and Milne, 1965; Greenwood and Milne, 1968; Muus, Brockett and Connelley, 1956; Urata, 1957), silver (Greenwood and Milne, 1968; Di Carlo and Redfern,

1947; Muus, Brockett and Connelley, 1956; Urata, 1957), copper (Di Carlo and Redfern, 1947; Greenwood and Milne, 1968; Muus, Brockett and Connelley, 1956; Urata, 1957) and lead (Di Carlo and Redfern, 1947; Greenwood and Milne, 1968; Urata, 1957). Generally, mercury was more inhibitory than copper or lead (Greenwood and Milne, 1968).

The α-amylases are known to produce a variety of low molecular weight maltodextrins. Early researchers believed that the action of α-amylase on starch was a random process resulting in a random selection of maltodextrins. When paper chromatographic techniques were used to detect the products of amylase digestion, it was observed that specific product patterns were characteristic of different α-amylases (Bird and Hopkins, 1954; Dube and Nordin, 1961; Pazur, French and Knapp, 1950; Robyt and French, 1963). Table 2 lists some of the predominant products that result from the action of various microbial amylases on starch.

Regulation of Amylase Synthesis

Enzyme synthesis in microorganisms is controlled by induction and catabolite repression. Although most of the mechanisms that have been studied concern the regulation of intracellular enzymes, several extracellular enzymes, including some amylases, have been shown to be controlled by

Table 2. Final hydrolytic products of various microbial amylases

Organism	Substrate	Main Products	References
Bacillus (alkaline amylase)	Starch	G_1, G_2, G_3	Horikoshi, 1971
Bacillus amyloliquefaciens	Starch	G_2, G_3, G_4, G_6	Welker and Campbell, 1967
Bacillus macerans	Starch	Schardinger dextrins	French, 1957
Bacillus subtilis SAC, NAR, NA64	Starch	G_1, G_2	Matsuzaki *et al.*, 1974
Bacillus subtilis NAT, NA20	Starch	G_2, G_3	Matsuzaki *et al.*, 1974
Bacteriodes amylophilus	Starch	$G_2, G_3, G_4, G_5, G_6, G_7$	McWethy and Hartman, 1977
Enterobacter aerogenes	Starch	G_6	Kainuma *et al.*, 1975
Streptococcus bovis	Starch	G_1, G_2, G_3	Walker and Hope, 1964
Streptococcus equinus	Starch	G_2, G_3, some G_1 and G_4	Boyer and Hartman, 1971
Bacillus polymyxa	Amylose	G_2, some G_1 and G_3	Robyt and French, 1964
Clostridium butyricum	Amylose	G_2, G_3 some G_1 and G_4	Hobson and MacPherson, 1952
Halobacterium halobium	Amylose	G_2, G_3 and G_1	Good and Hartman, 1970
Pseudomonas	Amylose	G_3, G_4	Kato *et al.*, 1975
Streptococcus	Amylose	G_2, G_3, some G_1 and G_4	Hobson and MacPherson, 1952
Streptococcus equinis	Amylose	G_2, G_3, G_4 some G_1	Boyer and Hartman, 1971
Bacillus polymyxa	Amylopectin	G_2	Marshall, 1974

Table 2 (Continued)

Organism	Substrate	Main Products	References
Bacillus polymyxa	Schardinger dextrins	G_2,G_3	Robyt and French, 1964
Bacillus subtilis NAT, NA20	G_4	G_2	Matsuzaki *et al.*, 1974
Pseudomonas	G_4	G_1,G_2	Kato *et al.*, 1975
Bacillus subtilis SAC, MAR, NA64	G_3 or G_4	G_1,G_2	Matsuzaki *et al.*, 1974
Bacillus subtilis	G_2,G_3 and G_4	No hydrolysis	Matsuzaki *et al.*, 1974
Clostridium butyricum	G_3	No hydrolysis	Walker and Hope, 1964
Pseudomonas	G_3	G_1	Kato *et al.*, 1975
Streptococcus bovis	G_3	Slowly to G_1,G_2	Walker and Hope, 1964

induction and repression mechanisms (Bull, 1972). The induction of extracellular amylase biosynthesis has been observed in several microorganisms. The production levels of glucoamylase and α-amylase of Clostridium acetobutylicum have been shown to be influenced by the type of carbohydrate in the growth medium. The glucoamylase was induced when glucose was present in the culture medium, while starch induced α-amylase (Ensley, McHugh and Barton, 1975). Induction of α-amylase production in C. acetobutylicum has also been observed by other researchers (Coleman and Elliott, 1962; Klein, 1963; Welker and Campbell, 1963b). Unlike the glucoamylase of Aspergillus niger (Barton, Georgi and Lineback, 1972), maltose did not stimulate glucoamylase production. Also, lactose and galactose, but not glucose, stimulated α-amylase production in B. subtilis in the absence of a nitrogen source (Coleman and Elliott, 1962). B. polymyxa amylase synthesis has been observed only when starch or starch-like products were present in the culture medium (Griffin and Fogarty, 1973). Studies of Pseudomonas saccharophila (Markovitz and Klein, 1955) indicated that α-amylase production could be induced by starch or maltose; however, it was later reported that these compounds probably contained contaminating oligosaccharides (Welker and Campbell, 1963a). Tomomura et al. (1961) demonstrated that

amylose, kojibiose, maltose, isomaltose and panose induced α-amylase formation in Aspergillus oryzae. This organism also possessed a transglucosidase which acted as a natural inducer of α-amylase synthesis in the presence of starch by producing isomaltose and panose.

It has been observed that media containing complex α-1,4-glucoside-containing raw materials (Bull, 1972; Priest, 1977; Saito and Yamamoto, 1975), such as maize, barley, wheat or malt, gave higher yields of α-amylase than defined media (Burbidge and Collier, 1968; Clary, Mitchell and Little, 1968; Nyiri, 1971; Saito and Yamamoto, 1975). Also, greater α-amylase production has been observed when the sole carbon source was starch, compared to yields obtained when glucose was the carbon source (Coleman and Grant, 1966; Fukumoto, Yamamoto and Tsuru, 1957; Welker and Campbell, 1963a). Since starch cannot penetrate the cell membrane because of its high molecular weight, the presumption that it is not directly involved in induction is prevalent (Ensley, McHugh and Barton, 1975; Priest, 1977; Saito and Yamamoto, 1975). It has been proposed that bacterial extracellular enzymes could be induced by the substrate attaching at a cell wall-membrane binding site (Priest, 1977). However, it is commonly believed that the microorganism produces a low level of constitutive enzyme which degrades the substrate releasing low molecular weight

products which induce further enzyme synthesis (Priest, 1977). Welker and Campbell (1963b) obtained evidence that supported this hypothesis; B. stearothermophilus formed α-amylase at a constant rate in the absence of maltose or maltodextrins, but in their presence an increased rate of amylase production was observed. Thus, it appeared that this enzyme has both constitutive and inducible properties. This phenomenon has also been reported for yeast β-glucosidase (MacQuillan, Winderman and Halvorson, 1960) and E. coli tryptophanase (Ng and Gartner, 1963). Constitutively produced α-amylase has been observed in B. amyloliquifaciens (Coleman, 1967), B. subtilis (Butterworth, Wang and Sinskey, 1970; Sekiguchi and Okada, 1972) and B. licheniformis (Meers, 1972).

A number of Bacillus strains have been reported that produce α-amylase when monosaccharides were used as the sole carbon source (Coleman and Elliott, 1962; Fukumoto, Yamamoto and Tsuru, 1957; Meers, 1972; Welker and Campbell, 1963a), indicating that this enzyme is constitutive in some bacterial strains.

In some instances, maltooligosaccharides, such as maltotriose (G_3), maltotetraose (G_4), maltopentaose (G_5), maltohexaose (G_6), and maltoheptaose (G_7), were superior to starch as inducers of B. licheniformis (Saito and Yamamoto, 1975). Maltotetraose was the most effective inducer of these low molecular weight maltodextrins (Welker and Campbell,

1963b). Oligosaccharides unrelated to starch, such as cellobiose and melibiose were poor inducers (Priest, 1977). Saito and Yamamoto (1975) observed that B. licheniformis cultures produced an inducer that accumulated extracellularly during growth and stimulated α-amylase synthesis in fresh cultures. It is possible that the inducing effect was caused by hydrolytic products of starch. Although maltodextrins induced amylase synthesis in some studies, maximum induction was obtained when raw starchy materials also were present in the growth medium (Bull, 1972; Priest, 1977; Saito and Yamamoto, 1975). Not only did these complex α1:4 glucosidic materials induce amylase production but they failed to express catabolite repression. Consequently, when B. subtilis α-amylase is commercially produced high concentrations of starch (8 to 12%) are used to attain high enzyme yields (Priest, 1977).

Other media constituents have also been studied as stimulators of enzyme synthesis. Cyclic adenosine monophosphate (AMP) reportedly increased the rate of α-amylase synthesis by a Bacillus sp. (Saito and Yamamoto, 1975; Yu-wei et al., 1973), but one investigator questioned if this was really induction because an α-amylase from Bacillus exhibited an increased reaction rate when the enzyme was assayed in the presence of cyclic AMP (Priest,

1975). Also, glucose repression of Bacillus subtilis α-amylase synthesis was not relieved by cyclic AMP (Priest, 1975).

Like cyclic AMP, gibberellic acid has been shown to induce the production of barley endosperm amylase, protease, acid phosphatase and adenosine triphosphatase (Earle and Galsky, 1971; Galsky and Lippincott, 1969; Gilbert and Galsky, 1972; Nickells, Schaefer and Galasky, 1971). Glucose repression of B. subtilis α-amylase was relieved by gibberellic acid (Yu-wei et al., 1973). They also found that B. subtilis α-amylase synthesis was prevented by Actinomycin D in the presence of gibberellic acid. Thus, they hypothesized that the action of gibberellic acid on α-amylase formation was mediated by cyclic AMP at the transcription level (Yu-wei et al., 1973).

Enzyme synthesis is also influenced by catabolite repression. Catabolite repression, the permanent repression of inducible or constitutive enzyme synthesis, has at least a contributory role in exoenzyme regulation (Priest, 1977). Some α-amylases are repressed by glucose (Glenn, 1976). Among these are the α-amylases of B. subtilis (Heineken and O'Connor, 1972; MacDonald-Green and Colarusso, 1964; Priest, 1975), B. licheniformis (Meers, 1972) and Vibrio parahaemolyticus (Tanaka and Iuchi, 1971). While repression

of V. parahaemolyticus α-amylase by glucose was relieved by cyclic AMP (Tanaka and Iuchi, 1971), the α-amylase of B. subtilis was not (Glenn, 1976). Fructose was reported to repress α-amylase synthesis by B. stearothermophilus (Welker and Campbell, 1963a), but not B. amyloliquefaciens (Coleman and Elliott, 1962). A dual control (induction and repression) mechanism was observed α-amylase synthesis by Bacteroides amylophilis (Bull, 1972) and B. licheniformis (Meers, 1972). Meers (1972) observed that nitrogen-limited cultures produced only a small amount of amylase, compared with the high levels observed in similar cultures that contained an excess of nitrogen. Despite research on induction and repression, the study of extracellular enzyme synthesis is basically an enigma (Bull, 1972).

Amylases of Ruminants

As herbivors, ruminants exist on a diet partially composed of starch. Thus, understanding their starch metabolism may be significant not only as biochemical research, but also from an economical viewpoint. Ruminants do not possess any salivary amylases (Campbell and Lasley, 1969). Consequently, it is commonly assumed that a major portion of the starch in the diet is metabolized by rumen microorganisms (Clary, Mitchell and Little, 1968). Starchy material that is not hydrolyzed in the rumen is

either hydrolyzed in the lower digestive tract or passes into the excreta.

Rumen microorganisms that produce amylase include: Bacteroides amylophilus, Bacteroides ruminicola, Bacteroides succinogenes, Butyrivibrio fibrisolvens, Clostridium lochheadii, Selenomonas ruminantium, Streptococcus bovis, Succinimonas amylolytica (Hungate, 1966) and Clostridium butyricum (Hobson and MacPherson, 1952; Walker and Hope, 1964). Of these enzymes produced by only Streptococcus bovis (Hobson and MacPherson, 1952; Walker, 1965; Walker and Hope, 1964), Clostridium butyricum (Hobson and MacPherson, 1952; Walker and Hope, 1964) and Bacteroides amylophilus (McWethy and Hartman, 1977) have been characterized. I. M. Robinson (personal communication, National Animal Disease Laboratory, Ames, IA, 1975) isolated a strain of Anaeroplasma bactoclasticum that possessed amylase activity. The study of this enzyme is the topic of this dissertation. Anaeroplasma blastoclostrium was selected because an anaerobic mycoplasma might produce amylases with unique properties.

MATERIALS AND METHODS

Organism

Anaeroplasma bactoclasticum strain 5 LA was obtained from Mr. I. M. Robinson, National Animal Disease Center, Ames, Iowa. This obligately anaerobic mycoplasma was isolated from an ovine rumen.

Cultures were grown anaerobically by using a modification of the Hungate technique (Hungate, 1966). Oxygen-free carbon dioxide was generated by passing hydrogen and carbon dioxide through a heated column of copper filings. Stock cultures were frozen and stored in media containing clarified rumen fluid at -70 C in a Kelvinator series 100 Ultra-cold freezer (Manitowoc, WS).

Media Preparation

All of the Media used in this study were prepared by using the following method: The medium was brought to a boil under an oxygen-free carbon dioxide atmosphere. The pH of the cooled medium was adjusted. Then the medium was gassed with oxygen-free carbon dioxide for 15 min. After autoclaving, sterile L-cysteine.HCl, sodium carbonate and penicillin G were added. The medium was gassed to a reduced state as demonstrated by the oxidation-reduction indicator resazurin.

Some media contained clarified rumen fluid. Bovine

rumen fluid was collected at slaughter from animals fed a diet of 70% cracked corn, 30% corn cobs, molasses and premix vitamins. The animals were also fed 4 lbs of alfalfa hay per day. Large quantities of rumen fluid were collected three times during this study. The rumen fluid of collection 1 was from a Hereford cross-bred, collection 2 was from a Simmental cross-bred, and collection 3 was from an Angus cross-bred. Following each collection a growth curve was performed to verify that media supplemented with each batch resulted in similar growth and amylase production patterns.

After slaughter, the bovine rumen was opened and its contents filtered through two layers of cheese cloth. The filtrate was sterilized in an autoclave at 15 lbs pressure for 30 min. After cooling, the rumen fluid was clarified by centrifugation at 46,000 x g with a Sharples super centrifuge (Sharples Corp., Philadelphia, PA). The fluid was heated in an autoclave as before, then was reclarified by centrifugation for 15 min at 16,300 x g and 4 C in a Sorvall RC2B Refrigerated Centrifuge (Norwalk, CN). The clarified rumen fluid was frozen and stored at approximately -15 C until needed. Immediately before use it was autoclaved and centrifuged again.

Growth Studies

Experiments varying medium constituents were performed to determine an optimal medium for amylase production. For preliminary studies of the basal media, 500-ml Florence flasks, each containing 200 ml of medium were used. All other constituent variation studies were performed by using 16 x 150 mm test tubes containing 10 ml of medium. Cultures were incubated at 35 C, and growth was monitored by absorbance with a Bausch and Lomb (Rochester, NY) Spectronic 20 Spectrophotometer.

Growth and amylase production in the optimal production medium were studied by using 200-ml cultures in 500-ml Florence flasks. Growth was monitored both by radioactive nucleic acid incorporation and by viable cell counts. The method of Byfield and Scherbaum (1966) was adopted to monitor ^{3}H-tyhmidine uptake. Duplicate 100-ml cultures containing 120 µl of ^{3}H-thymidine (New England Nuclear, Boston, MA; 0.50 mCi/ml) were used. Each culture was seeded from a different inoculum. At each sample time duplicate 74-µl samples were applied to paper discs (Whatman 3MM, 2.3-cm scintillation pads) for heterogeneous counting. The discs were treated as described by Byfield and Scherbaum (1966). Samples were counted by using a Beckman Liquid Scintillation System LS-100 (Palo Alto, CA). Both the channels ratio and

external standard methods of quench correction were utilized. Samples were counted to 2% relative standard deviation. Viable-cell counts were determined anaerobically by using roller bottles (Astell Laboratory Service Co., Ltd., London, England) and an electric bottle spinner (Astell Laboratory Service Co., Ltd.). The agar medium used for viable-cell counts was developed from the formulation of the production medium. Duplicate counts were made for each sampling time. The bottles were incubated at 35 C for 48-60 hours and counted.

Amylase Assays

Several assays were used to detect amylase activity. These included the blue-value assay of Robyt and Whelan (1968). This assay measures the decrease of blue staining of the substrate with iodine caused by enzyme action. The blue-value was defined as D-D'/D, where D was the absorbance of the substrate-iodine complex without enzyme present and D' was the absorbance of the enzyme digest.

The reducing-value assay of Robyt and Whelan (1968) was also used as a measure of amylase activity. As standards, dilutions of maltose ranging from 50 to 1000 µg/ml were assayed. Reducing-value amylase units were expressed as µmoles of glycosidic bonds cleaved by using the following equation:

$$U = \frac{\mu g \text{ maltose/ml/min}}{342}$$

For both of these assays a substrate of 1% soluble starch (Fisher Scientific Co., Pittsburgh, PA) in an appropriate buffer was utilized; buffer compositions are described in RESULTS.

The disc-plate amylase assay of Stark et al. (1953) was modified for this study. A survey of buffer systems, various molarities and pH variations resulted in an optimal disc-plate assay system for this Anaeroplasma enzyme. Thus, the use of petri plates containing 20 ml of starch agar (0.2% soluble starch) (Fisher Scientific Co., Pittsburgh, PA), 1mM $CaCl_2$ and 1.0% agar in 0.01 M piperazine-N,N'-Bis (ethanesulfonic acid)(PIPES; Calbiochem, LaJolla, CA) at pH 6.0 was used for the remainder of this study.

Each sample was assayed on two starch agar plates by using four discs per plate. Each filter paper disc (12.7 mm diameter, Schleicher and Schuell Co., Keene, NH) was saturated with the sample, then touched to the side of the sample container to drain any excess liquid before being placed on the plate. Plates were incubated upright at 42 C for 8 h. Following incubation, the discs were removed and the plates flooded with an iodine solution (3.0% KI and 0.3% I_2). The excess iodine solution was poured off and

clear zones of starch hydrolysis were measured by using a Fisher-Lilly zone reader (Fisher Scientific Co., Pittsburgh, PA). Thus, eight replications were measured for each sample.

Protein Determination

Protein concentrations were determined by using the method of Lowry et al. (1951). Dilutions of bovine serum albumin (Sigma Chemical Co., St. Louis, MO), ranging from 50 to 1000 µg/ml, were used as standards.

Extracellular Amylase Purification

For purification, Anaeroplasma bactoclasticum amylase was produced in 9-l quantities in 12-l pyrex, flat-bottom Florence flasks. Each culture was seeded with a 10% inoculum. Because the mycoplasma produced gas it was necessary to bubble oxygen-free carbon dioxide through the culture throughout growth. This procedure kept the culture anaerobic and since the flask was vented, an increase of pressure within the vessel was avoided. Cultures were incubated for 24 h in a 40 C water bath, resulting in a culture temperature of 35-37 C. The spent medium was subjected to centrifugation at 4 C for 15 min at 16,300 x g to obtain the cell-free broth containing extracellular amylase. The purification procedure developed is discussed in RESULTS.

Paper Chromatography

Chromatograms were prepared from 30 x 40 cm pieces of Whatman No. 1 filter paper. Samples were spotted on the chromatograms in 20-µl aliquots. Spots were placed 2.5 cm from the bottom, 3.0 cm from the sides and 2.5 cm from adjacent spots. The chromatograms were stapled to form cylinders and placed in 15 x 45 cm pyrex cylindrical tanks containing 250 ml of 70% n-propanol as a solvent. Three ascents of each chromatogram were made at 35 C. The chromatograms were thoroughly dried between each ascent. After the third ascent, the chromatograms were dried and then were developed by using a silver nitrate dip method (Robyt and French, 1963). Standards containing maltodextrins G_1 through G_7 or G_1 through G_{15} (obtained from Dr. J. Robyt, Iowa State University, Ames, IA) were compared to enzyme digest maltodextrins.

Disc-gel Electrophoresis

The disc-gel electrophoresis and staining procedures described by Ortec, Inc. (Application Note 32A: Techniques for High Resolution Electrophoresis, 1973) were implemented by using a Buchler Instruments (Fort Lee, NJ) disc-gel electrophoresis apparatus. This study utilized 3% acrylimide stacking gels and 8% acrylamide separating gels because this step gradient is commonly used for lipoproteins.

(Certain data, described in RESULTS, led me to believe that the amylase activity was associated with a lipoprotein). The acrylamide (Sigma Chemical Co., St. Louis, MO) gels contained a tris-citrate buffer, pH 9.0 (0.15 M and 0.75 M) system, while the electrophoresis buffer was 0.065 M tris-borate, pH 9.0. Enzyme samples, in a sample gel containing the components of the 3% acrylamide layer, were layered above the polymerized gel in 6 (I.D.) x 170 mm tubes. Approximately 200 µg of protein were applied per tube. Electrophoresis was accomplished at a current of 0.5 mA per tube by using a Voltage and Current Regulated DC Power Supply (Buchler Instruments). Electrophoresis was carried out at 4 C. In other experiments, 8% acrylamide separating gels were utilized, as discussed above, at a current of 5 mA per tube.

Duplicate gels were stained for lipoproteins, glycoproteins, and proteins by using stains described by Ortec (Application Note 32A: Techniques for High Resolution Electrophoresis, 1973). Lipoproteins were prestained with a sudan black stain, while glycoproteins and proteins were stained after electophoresis by the periodic acid-Schiff stain and the coomassie blue stain, respectively.

Bands of amylase activity were visualized as follows: After electrophoresis, gels were submerged in 0.01 M PIPES buffer, pH 6.0 and containing 1mM $CaCl_2$ and 2% soluble starch,

and were refrigerated overnight. The gels were then incubated in the starch solution at 42 C for 30 min. Following decantation of the starch solution, the gels were immersed in iodine solution (3.0% KI plus 0.3% I_2). Bands of clearing denoted amylase activity. This method was a modification of one described by Isono (1970).

Molecular Weight

Early studies to find a general molecular weight range of the enzyme were performed by ultrafiltration with an Amicon Diaflo Ultrafiltration Apparatus (Amicon Corp., Lexington, MA). A series of Diaflo Ultrafilters (Amicon Corp.) were used to separate different molecular weight molecules. A cell-free supernatant was filtered by using the following series of filters: XM-100A, XM-50, PM-30 and PM-10. These filters reportedly yield the following approximate molecular weight ranges: greater than 100,000, 50,000-100,000, 30,000-50,000, 10,000-30,000, and less than 10,000 daltons, respectively. A flow chart of this procedure appears in Figure 1. Nitrogen was used to provide the proper pressure. Pressures used corresponded to the recommended pressures for the respective filters (Amicon Publication 1-101 D: Instructions Diaflo Ultrafilters). Ultrafiltration was performed at 4C. Further molecular weight determination of the partially purified amylase was performed by using sodium

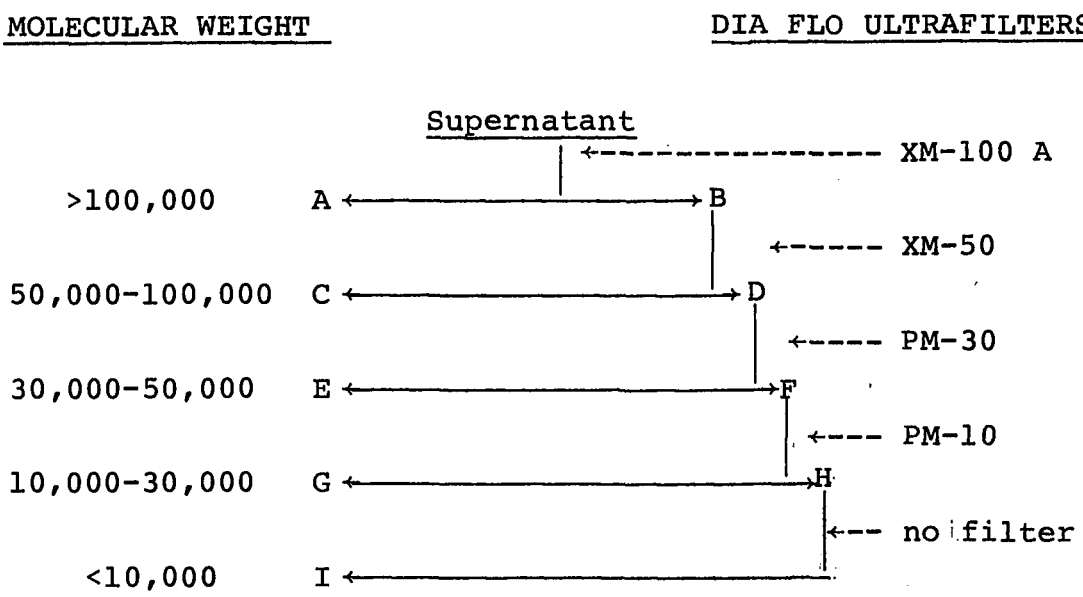


Figure 1. Flow chart of ultrafiltration to separate amylase by molecular weight

dodecyl sulfate disc-gel electrophoresis. The procedures outlined by Ortec, Inc. (Application Note 32A: Techniques for High Resolution Electrophoresis, 1973) were used.

RESULTS

Disc-plate Assay

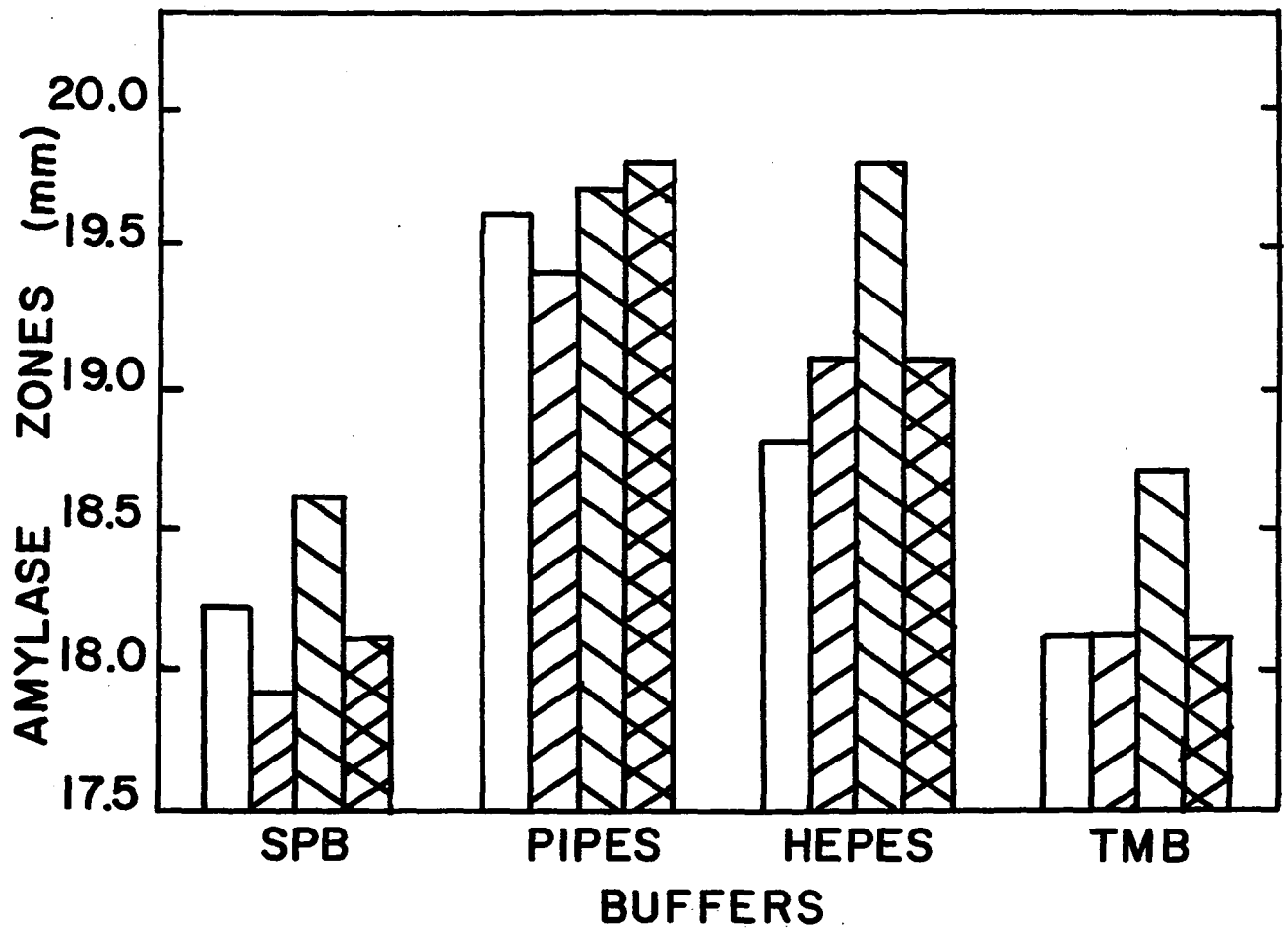
Before studying an enzyme it is essential to have a method of accurately assaying enzyme activity. As discussed in MATERIALS AND METHODS, three assays were used to monitor amylase throughout this study. The disc-plate assay of Stark et al. (1953) was modified resulting in a system optimal for Anaeroplasma amylase detection.

The disc-plate amylase assay was analyzed by using various buffers, pHs, and supplementary ions. The following buffers were tested: (i) M/15 Sorenson's phosphate (SPB), (ii) 0.2 M tris-malate (TMB), (iii) 0.01 M piperzine N,N'-Bis (2-ethane sulfonic acid)(PIPES), and (iv) 0.01 M N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid (HEPES). These buffers were studied for various reasons. SPB was the buffer originally used for this assay; TMB was used because tris buffers are frequently used in enzyme research. The two organic buffers were chosen because of their negligible sequestering activity. A pH range of 5.0 to 7.5 was studied. Calcium ions (Boyer and Hartman, 1971; Fischer and Stein, 1950; Greenwood and Milne, 1968; Manning and Campbell, 1961) and sodium chloride (Boyer and Hartman, 1971; Good and Hartman, 1970) have previously been reported to increase amylase activity. Consequently, assay media

containing 1 mM $CaCl_2$, 1 mM NaCl or a combination of these two salts were compared to the corresponding assay medium that did not contain these supplementary ions. Other than these variations the procedures of Stark et al. (1953) were followed.

Like several other microbial amylases, as discussed in LITERATURE REVIEW, the Anaeroplasma amylase had an optimum pH of 6.0. Figure 2 is a comparison of the four buffer systems containing the various ion combinations studied at pH 6.0. Amylase activity was greater when the enzyme was assayed in the two organic buffers as compared to SPB and TMB. In PIPES the amylase exhibited more uniform zone sizes than in HEPES when the various supplementary ions were examined. The addition of NaCl did not significantly effect enzyme activity, except when the enzyme was suspended in HEPES buffer. The addition of $CaCl_2$ resulted in maximum amylase activity for all four buffers. In HEPES, SPB and TMB the addition of both salts gave results similar to those obtained when NaCl alone was added to the buffer. This was surprising because it was predicted that approximately the same enzyme activity would be observed when both $CaCl_2$ and NaCl were added as when only $CaCl_2$ was added. The expected results were observed when PIPES buffer was used. Optimal amylase activity was attained when $CaCl_2$ was added

Figure 2. Comparison of Anaeroplasma bactoclasticum amylase activity by the disc-plate assay (various buffers at pH 6.0 were studied including M/15 Sorenson's phosphate (SBP), 0.01 M HEPES, and 0.02 M Tris-malate (TMB). The buffers were supplemented with 1 mM NaCl (▨), 1 mM $CaCl_2$ (▧), a combination of both salts (▩) or no salts were added (□)

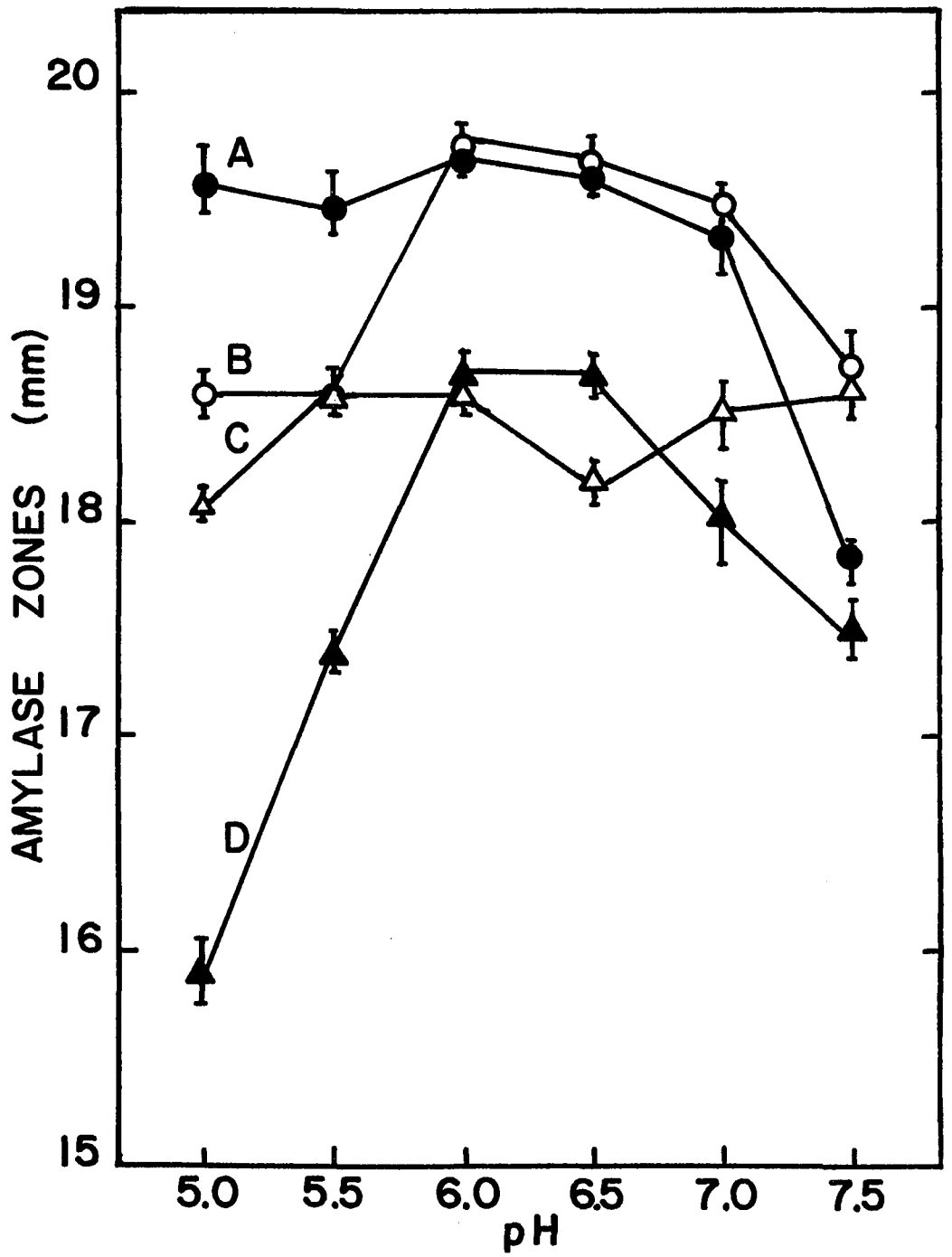

AMYLASE ZONES (mm)
20.0
19.5
19.0
18.5
18.0
17.5
SPB
PIPES
HEPES
TMB
BUFFERS

to either PIPES or HEPES.

Figure 3 compares the four buffers, each containing 1 mM $CaCl_2$, over the pH range 5.0 to 7.5. In PIPES, HEPES and SPB buffers the enzyme maintained somewhat uniform amylase zone sizes over the pH range 6.0 to 7.0; whereas this was evident only at pH 6.0 and 6.5 for TMB. Smaller amylase zones were observed for PIPES above pH 7 and HEPES below pH 6.0, while in SPB the enzyme exhibited rather constant amylase zone sizes throughout the 5.0 to 7.5 pH range. TMB, however, only gave large zones at pH 6.0 and 6.5. Zone sizes of amylase in SPB and TMB were considerably smaller than those of the two organic buffers throughout the pH range. PIPES maintained maximum amylase activity over the pH range 5.0 to 7.0. Thus, PIPES was considered to be superior to HEPES, SPB and TMB for amylase detection. Consequently, the disc-plate assay was modified to include 0.01 M PIPES buffer containing 1 mM $CaCl_2$ at pH 6.0.

These data indicated decreased amylase activity when the enzyme was assayed in SPB as compared to PIPES. This raised questions concerning the possibility that Anaeroplasma bactoclasticum amylase was inhibited by phosphate. Consequently, the following buffer systems at pH 6.0, all containing 1 mM $CaCl_2$ were studied: (i) PIPES, (ii) SPB and (iii) a combination of PIPES and SPB. The results, averages

Figure 3. Comparison of the effect of pH on Anaeroplasma bactoclasticum amylase in various assay buffers containing 1 mM $CaCl_2$. (the following buffers are illustrated: line A, 0.01 M PIPES; line B, 0.01 M HEPES; line C, M/15 Sorenson's phosphate, and line D, 0.2 M tris-malate)


20
19
18
17
16
15
AMYLASE ZONES (mm)
A
B
C
D
5.0
5.5
6.0
6.5
7.0
7.5
pH

of 16 replications, showed that PIPES, SPB and the combination gave zone sizes of 19.1, 18.1 and 18.0 mm, respectively. Thus, the phosphate buffer apparently interfered with the expression of amylase activity.

Development of Production Medium

Because Anaeroplasma bactoclasticum is a rumen microorganism, the development of an optimal production medium was based on media developed for rumen bacteria. The initial growth media tested were modifications of clarified rumen fluid broth (Robinson, 1973) and a medium without rumen fluid, medium-10 (Caldwell and Bryant, 1966). The compositions of these modified media appear in Tables 3 and 4. These two media, containing either 0.2% soluble starch (Fisher Scientific Co., Fair Lawn, NJ) or 1% dry diamalt diastatic (Standard Brands Incorporated, New York, NY) as carbon sources, were used to compare amylase production in rumen fluid based media with that in media not containing rumen fluid (Figure 4). These data demonstrated that media containing rumen fluid were superior to media without rumen fluid with respect to amylase production. It was also observed (data not shown) that penicillin G had no effect on amylase production. Therefore, basal media containing clarified rumen fluid and penicillin G were used in all

Table 3. Components of modified clarified rumen fluid broth[a]

Component	Percentage
Clarified rumen fluid	40.0
Carbon source[b]	-
Mineral solution A[c]	3.75
Mineral solution B[c]	3.75
Resazurin	0.0001
Na_2CO_3	0.4
Cysteine·HCl	0.05
Deionized distilled water	52.05

[a]A modification of clarified rumen fluid broth of Robinson (1973); the final pH was 6.8.

[b]A variety of carbon sources at various concentrations was studied.

[c]Mineral stock solution A contained NaCl, 1.2%; $(NH_4)_2SO_4$, 1.2%; KH_2PO_4, 0.6%; $CaCl_2$, 0.12%; and $MgSO_4$, 0.25%. Mineral solution B contained K_2HPO_4, 0.5%.

Table 4. Components of modified medium-10[a]

Component	Percentage
Carbon source[b]	-
Mineral solution A[c]	3.75
Mineral solution B[c]	3.75
Volatile fatty acid mixture[d]	0.31
Trypticase	0.2
Yeast extract	0.05
Cholesterol[e]	0.002
Lecithin	0.1
Resazurin	0.0001
Na_2CO_3	0.4
Cysteine·HCl	0.05
Deionized distilled water	91.4

[a]A modification of Medium-10 of Caldwell and Bryant (1966); final pH was 6.8.

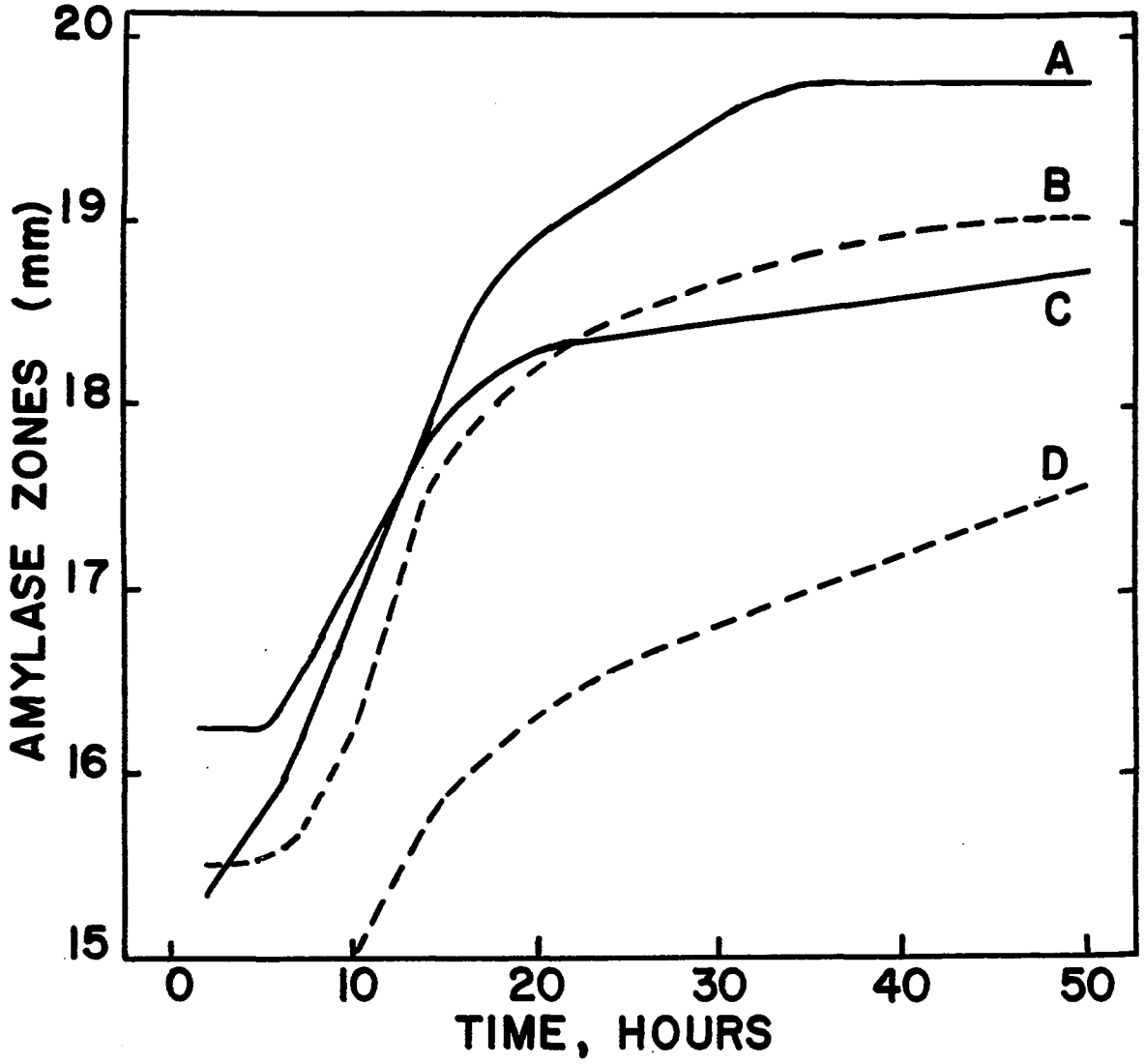
[b]A variety of carbon sources at various concentrations were studied.

[c]The mineral solutions were those described in Table 3.

[d]The volatile fatty acid mixture contained: acetate, 53.6%; propionate, 21.3%; n-butyrate, 12.9%; iso-butyrate, 2.6%; n-valerate, 3.2%; iso-valerate, 3.2% and DL-α-methylbutyrate, 3.2%.

[e]A 10 mg/ml stock solution of cholesterol was prepared by dissolving the cholesterol in 5 ml of ethanol. Deionized distilled water was added to make 100 ml.

Figure 4. Comparison of Anaeroplasma bactoclasticum amylase production in rumen fluid and nonrumen fluid based media (|----| represents amylase from cultures grown in nonrumen fluid based media, modified medium 10 base; while |——| represents amylase from cultures grown in clarified rumen fluid based media. Lines A and B represent media containing 1% dry diamalt diastatic, and lines C and D represent media containing 0.2% soluble starch as carbon sources)

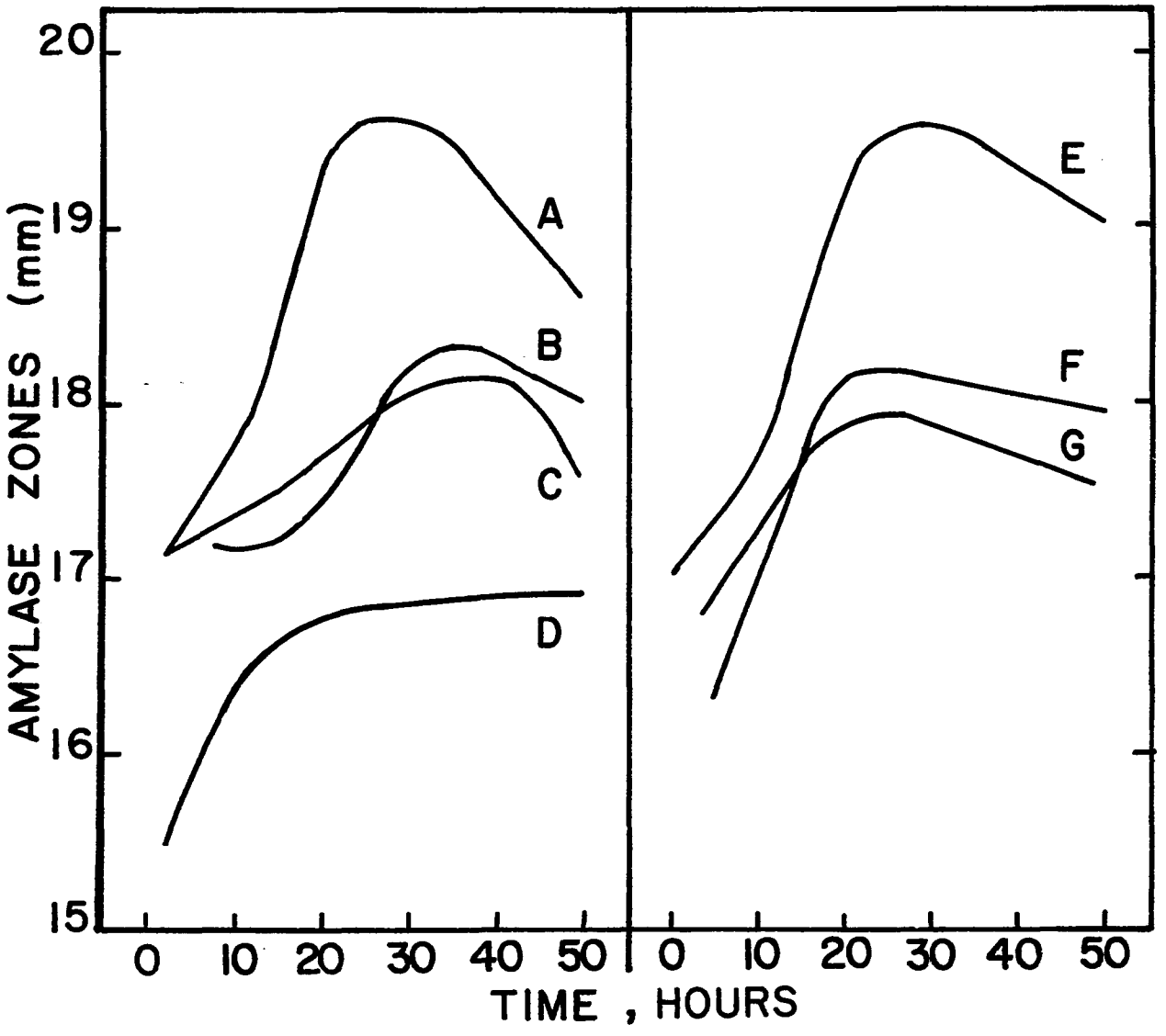

20
19
18
17
16
15
AMYLASE ZONES (mm)
A
B
C
D
0
10
20
30
40
50
TIME, HOURS

further studies.

Results of the effect of basal medium variations on amylase production appear in Figure 5. All of these media contained 3.75% of the mineral solutions (see Table 3 for components of the mineral solutions), 0.002% cholesterol, 0.001% resazurin, 1% dry diamalt diastatic, 0.4% Na_2CO_3, 0.05% cysteine·HCl, 1000 U/ml penicillin G and, unless otherwise indicated, 40% clarified rumen fluid. Medium A, a control, contained only the components listed above. Medium B contained 10% fetal calf serum and 5% Eagles Basal Medium (Vera and Dumoff, 1974), while Medium C was only supplemented with 10% fetal calf serum. These components were included because they had previously been observed to enhance growth of aerobic mycoplasmas (Maniloff and Morowitz, 1972; Razin, 1973). Medium D was similar to modified medium-10 in that it contained 0.2% trypticase, 0.05% yeast extract and 0.1% lecithin. Medium E was supplemented with 0.01 M PIPES. Medium F was identical to the control, except the amount of clarified rumen fluid was decreased to 20%. In medium G only 10% clarified rumen fluid was added.

Figure 5 compares amylase production in these media. Growth was also monitored (data not shown). In Medium A, the control, Anaeroplasma produced the greatest amount of

Figure 5. Comparison of Anaeroplasma bactoclasticum amylase production in different basal media (medium A, the control, contained 7.5% mineral solution, 0.002% cholesterol, 0.001% resazurin, 1.0% dry diamalt diastatic, 0.4% Na_2CO_3, 0.05% cysteine·HCl, 1000 U/ml penicillin G and 40% clarified rumen fluid. The other media contained these constituents and were supplemented as follows: Medium B, 10% fetal calf serum; Medium D, 0.2% trypticase, 0.05% yeast extract and 0.1% lecithin; Medium E, 0.01 M PIPES; Medium F, the rumen fluid content was decreased to 20%; Medium G, the rumen fluid content was decreased to 10%)


AMYLASE ZONES (mm)
20
19
18
17
16
15
A
B
C
D
E
F
G
0
10
20
30
40
50
TIME , HOURS

amylase reaching a maximum zone size of 19.7 mm. When PIPES was added (medium E), amylase production was not affected. Besides amylase production, the growth rate was also not affected. The addition of Eagle's basal medium and/or fetal calf serum not only decreased the amount of amylase produced, but also adversely affected growth. A maximum amylase zone size of 17.1 mm was produced when yeast extract, trypticase and lecithin were added to the medium. When Anaeroplasma was grown in this medium, medium D, the least amylase activity was observed of all seven basal media.

Robinson (1973) reported that 40% clarified rumen fluid was the optimum percentage for growth of Anaeroplasma. The effect on amylase production of clarified rumen fluid concentration was demonstrated by comparing media A, F and G. Medium A, containing 40% clarified rumen fluid, resulted in the greatest amylase production. As the percentage of clarified rumen fluid in the medium was decreased from 40% to 10%, the amount of amylase produced also decreased. In all seven media no appreciable pH change was observed over the entire growth period.

A preliminary experiment, testing amylase induction, was performed. The following carbon sources were examined at concentrations of 0.2, 0.5 and 1.0%: maltose (J. T. Baker Co., Phillipsburg, NJ), maltrin-10 (maltodextrin mixture with an

average degree of polymerization of 10, Grain Processing Corp., Muscatine, IA), G_{40} (maltodextrin mixture with an average degree of polymerization of 40, J. F. Robyt, Iowa State University, Ames, IA), Texrez Extender G (an α1:4 glycoside with an average degree of polymerization of 980, Stein, Hall and Co., New York, NY), amylopectin (National Biochemical Corp., Cleveland, OH), soluble starch (Fisher Scientific Co., Fair Lawn, NJ), shellfish glycogen (Mann Research Laboratories, Inc., New York, NY), dextran (Sigma Chemical Co., St. Louis, MO), dry diamalt diastatic (Standard Brands Inc., New York, NY), potato starch granules (J. F. Robyt, Iowa State University, Ames, IA) and maize starch granules (J. F. Robyt, Iowa State University, Ames, IA). For each carbon source the greatest activity was observed when a concentration of 1.0% was used. Of these carbohydrates the least activity was observed when maltose (G_2) was the sole carbon source. Zone sizes of 16.1, 16.4 and 16.8 mm were observed for 0.2, 0.5 and 1.0% maltose, respectively.

As discussed in the LITERATURE REVIEW, media containing complex α1:4 glucoside-containing raw materials have been shown to result in higher amylase yields for other microorganisms (Bull, 1972; Priest, 1977; Saito and Yamamoto, 1975). Thus, it was not surprising that the largest amylase zone sizes were observed when 1% soluble starch, maize starch granules or glycogen were used as sole carbon sources.

When these carbon sources were used, Anaeroplasma produced amylase zones with diameters of 18.8, 19.0 and 19.1 mm, respectively.

Further investigation of carbon source induction was performed by using 0.1, 0.25, 0.5 and 0.75% concentrations of the following complex carbon sources: Dextrin (Difco Laboratories, Detroit, MI) soluble starch, shellfish glycogen, potato starch granules, rice starch (BDH Chemicals Ltd., Poole, England), wheat starch (BDH Chemicals Ltd., Poole, England), maize starch (BDH Chemicals Ltd., Poole, England), maize starch granules and commercial Argo corn starch (Best Foods, Englewood Cliffs, NJ). These carbon sources were added to the basal medium previously described and also two other basal media.

Suzuki, Tsuji and Abe (1976) observed that 1.0% peptone added to their medium for maltase production by thermophilic Bacillus sp. KP 1035 stimulated enzyme synthesis. Besides utilizing induction mechanisms, extracellular bacterial enzyme quantities have been increased by facilitating secretion with the addition of 0.1% Tween-80 (polyethylene glycol sorbitan monooleate; Reese and Maguire, 1969; Umesaki, Kawai and Mutai, 1977). Therefore, the above carbon sources were evaluated not only in the basal medium previously described, but also in the basal medium containing

1.0% Bacto-peotone (Difco Laboratories, Detroit, MI) and the basal medium containing 0.1% Tween-80 (Fisher Scientific Co., Fair Lawn, NJ).

These data are shown in Table 5. A trend of increasing amylase production was observed as the carbon source concentration increased. There were a few exceptions where larger amylase zones were observed from cultures containing 0.5% carbohydrates as compared to the corresponding medium containing 0.75% carbohydrate. In these instances, the difference in zone size was small. In fact, when the standard deviations were considered approximately the same levels of amylase production occurred at both the 0.5 and 0.75% concentrations.

The addition of Tween-80 to the medium did not increase the amount of enzyme detected. In several cases, such as when maize starch, wheat starch and shellfish glycogen were used at concentrations of 0.75%, amylase zones were at least 1 mm smaller when Tween-80 was added. On the other hand, the addition of 1.0% peptone greatly enhanced enzyme production, resulting in at least 1 mm increase in zone size. Exceptions included media containing glycogen as a carbon source. When this carbohydrate was used the addition of peptone did not increase amylase production at any of the four concentration levels studied. The effects of peptone on amylase production were greatest when the more

Table 5. Effect of carbon source, Tween-80 and Bacto-peptone on Anaeroplasma bactoclasticum amylase production

Carbon source	Percent concentration	Average amylase zones (mm)[a]		
		Basal medium	Basal medium + Tween-80	Basal medium + peptone
Dextrin	0.1	16.7	16.9	18.3
	0.25	18.1	18.0	18.9
	0.5	18.3	18.2	19.0
	0.75	18.0	18.1	19.7
Soluble starch	0.1	17.5	16.5	18.1
	0.25	17.8	16.9	18.0
	0.5	18.0	17.7	18.2
	0.75	18.0	17.9	19.4
Glycogen	0.1	17.3	17.8	17.7
	0.25	17.8	18.1	18.2
	0.5	19.1	18.5	18.5
	0.75	19.1	18.1	19.2
Potato starch granules	0.1	16.8	17.9	18.0
	0.25	18.4	18.4	18.5
	0.5	18.5	18.5	19.4
	0.75	18.9	18.6	20.0
Rice starch	0.1	17.3	17.6	18.1
	0.25	17.9	17.3	19.3
	0.5	18.7	18.1	19.8
	0.75	19.0	18.5	20.2
Wheat starch	0.1	17.7	17.6	18.3
	0.25	18.2	17.7	19.1
	0.5	18.4	17.8	19.6
	0.75	19.9	18.4	20.2
Maize starch	0.1	17.3	17.6	18.2
	0.25	18.2	17.8	19.8
	0.5	18.7	18.2	20.0
	0.75	19.1	18.0	20.8

[a]The disc-plate amylase assay was used. The numbers represent averages of 16 replications. The average standard deviation for all carbohydrates tested was 0.3 mm.

Table 5 (Continued)

Carbon source	Percent concentration	Average amylase zones (mm)[a]		
		Basal medium	Basal medium + Tween-80	Basal medium + peptone
Maize starch granules	0.1	16.9	17.5	18.1
	0.25	17.6	18.4	19.1
	0.5	18.4	18.6	19.6
	0.75	18.4	18.6	19.5
Argo corn starch	0.1	17.2	18.1	17.8
	0.25	18.2	18.5	18.9
	0.5	18.4	18.8	19.4
	0.75	17.9	18.7	19.8

complex raw starches were used as carbon sources.

Three types of corn starch were studied. Comparable results were obtained from media containing maize starch granules and Argo corn starch. Maize starch (BDH Chemicals Ltd., Poole, England) induced amylase activity to a greater extent. Of all carbohydrates studied the most amylase detected (a zone size of 20.8 mm) occurred when Anaeroplasma bactoclasticum 5LA was grown in a basal medium containing 1.0% peptone and 0.75% maize starch.

Further experimentation (data not shown) demonstrated that greater amylase production was not attained when maize starch was used in combination with either wheat or rice starch. Also, agitation during growth did not increase amylase production. Maize starch concentrations of 0.1, 0.25, 0.5, 0.6, 0.75, 0.8 and 1.0% were examined. The

results of these comparisons (data not shown) revealed that 0.75% was the optimum concentration of maize starch for amylase production by this organism.

Coleman and Elliott (1962) observed that Fe^{+3} ions increased amylase production from Bacillus subtilis; the optimum concentration of $FeCl_3$ ranged from 40 to 100 mM. When 40 mM of $FeCl_3$ was added to the clarified rumen fluid basal medium containing 1% peptone and 0.75% maize starch a globular precipitate was formed. Therefore, the concentration was decreased to 3mM (0.045%) because this was the highest level of other minerals in the medium. At 12 h a large amount of black precipitate was evident in the culture. As growth proceeded to 36 h the amount of precipitate decreased, indicating that this substance was being solubilized. It is known that $FeCl_3$ combines with H_2S to form a black precipitate, FeS. Thus, it is possible that the black substance observed indicated H_2S production. Concerning the effect on amylase, the addition of $FeCl_3$ did not enhance or inhibit the production of this enzyme.

Calcium ions have previously been reported to increase amylase activity (Boyer and Hartman, 1971; Fischer and Stein, 1960; Greenwood and Milne, 1968). Therefore, the possibility of increasing amylase production by adding 1 mM $CaCl_2$ to the medium was tested. When the $CaCl_2$ concentration was

increased to this level no substantial increase in amylase activity was detected with either the disc-plate or blue value assays. However, the reducing value assay showed an increase from 1.6 to 2.4 U/ml. It was later shown that the reducing value assay was unreliable for this system because of interference from the basal medium; these data will be discussed later. Calcium ions have been shown to increase the stability of other amylases (Fischer and Stein, 1960; Greenwood and Milne, 1968; Kainuma et al., 1975). Therefore, it seemed expedient to increase the $CaCl_2$ concentration to 1 mM. After this extensive survey, involving 194 different formulations, an optimal production medium (PM) was developed. This medium is described in Table 6. To determine some of the major unknown organic components of this medium, the production medium was analyzed by gas-liquid chromatography (Hewlett-Packard Model 5754B, Cupertino, CA). These results are in Table 7.

Growth and amylase production were studied in the production medium. Viable-cell counts were obtained by using an agar medium similar to PM. This medium, amylose azure rumen fluid agar, appears in Table 8. Amylose Azure (Calbiochem, Los Angeles, CA), a blue water-insoluble amylose, is amylose crosslinked with the dye 1,4-butanediol glycedyl ether. When extracellular amylase diffused through

Table 6. *Anaeroplasma bactoclasticum* amylase production medium[a]

Component	Percentage
Clarified rumen fluid	40.0
Mineral solution A[b]	3.75
Mineral solution B[b]	3.75
Maize starch	0.75
Bacto-peptone	1.0
Cholesterol[c]	0.002
Resazurin	0.0001
Na_2CO_3	0.4
Cysteine·HCl	0.05
Penicillin G[d]	0.0006
Deionized distilled water	50.3

[a]The final pH was 6.8.

[b]The mineral solutions were those found in Table 3; however, the $CaCl_2$ concentration was increased to 0.24%.

[c]Cholesterol was prepared as described in Table 4.

[d]The final concentration of penicillin G was 1000 U/ml.

Table 7. Some organic components of Anaeroplasma bactoclasticum amylase production medium, as determined by gas-liquid chromatography

Component	μM/ml
Ethanol	13.0
Acetate	34.7
Propionate	5.1
Iso-butyrate	0.1
Butyrate	5.4
Iso-valerate	0.9
Valerate	1.8
Caproate	0.3
Phenylacetate	3.9
Succinate	0.4

Table 8. Amylose azure rumen fluid agar[a]

Component	Percentage
Clarified rumen fluid	40.0
Mineral solution A[b]	3.75
Mineral solution B[b]	3.75
Bacto-peptone	1.0
Soluble starch	0.05

[a]The final pH was 6.8.

[b]The mineral solutions were those found in Table 3; however, the $CaCl_2$ concentration was increased to 0.24%.

Table 8 (Continued)

Component	Percentage
Amylose azure	0.5
Cholesterol[c]	0.002
Na_2CO_3	0.4
Cysteine·HCl	0.05
Agar	1.5
Penicillin G[d]	0.0006
Deionized distilled water	49.0

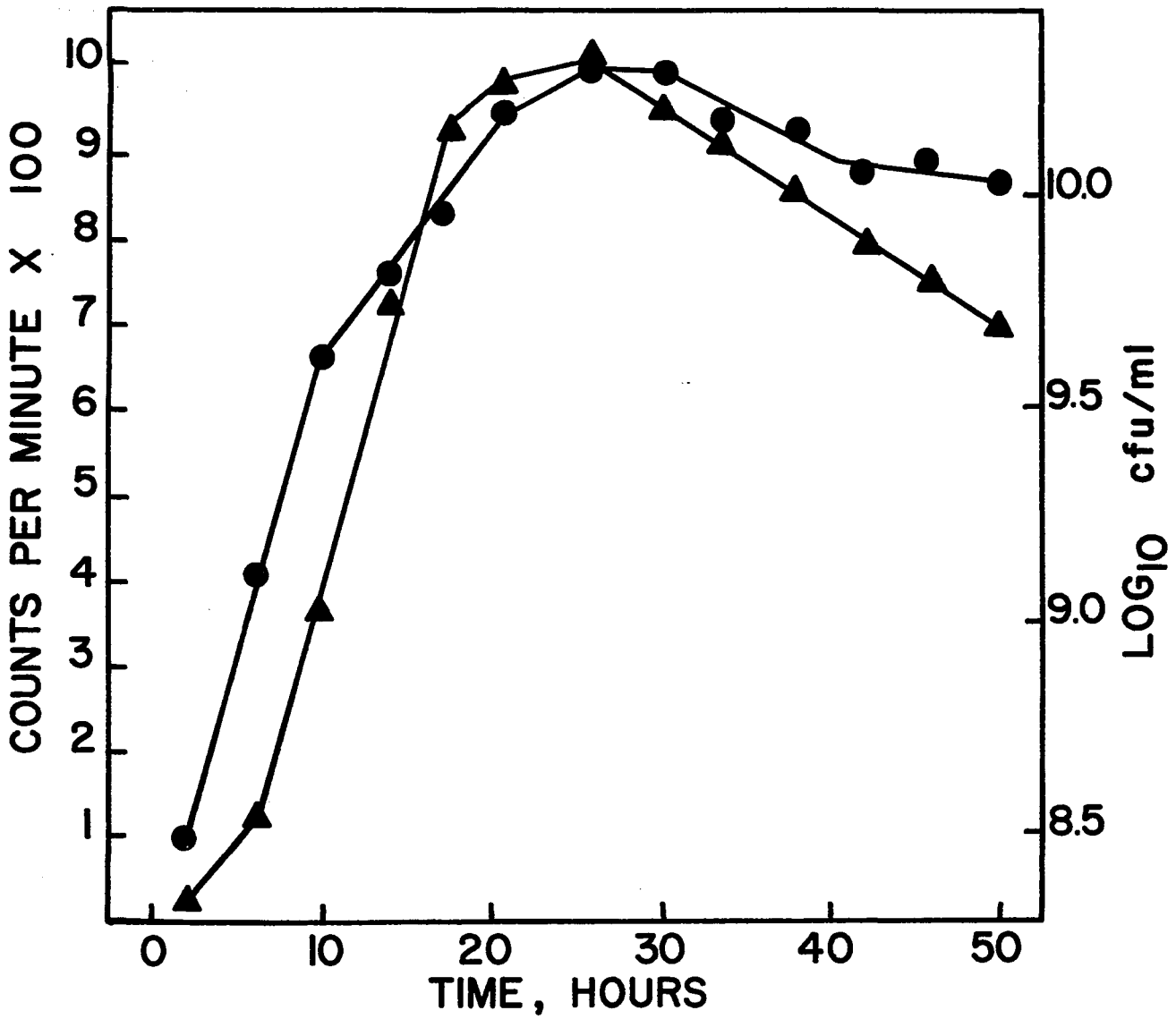
[c]Cholesterol was prepared as described in Table 4.

[d]The final concentration of penicillin G was 1000 v/ml.

this agar and amylose was hydrolyzed, soluble products were produced resulting in a zone of clearing around amylolytic colonies. The prereduced diluent used in growth studies contained 3.75% of each mineral solution, 0.42% volatile fatty acid mixture, 0.4% Na_2CO_3, and 0.05% cytstine·HCl. Duplicate roll tube viable counts were made at 4-h intervals throughout the growth period. Data, averaged from two experiments, appear in Figure 6.

Turbidity has been shown to be inadequate for accurately measuring mycoplasmal growth (Maniloff and Morowitz, 1972). Therefore, growth was also monitored by ^3H-thymidine uptake by using the procedure of Byfield and Scherbaum (1966). Duplicate samples were taken at 4 h intervals.

Figure 6. Growth study of Anaeroplasma bactoclasticum in amylase production medium (growth was monitored by viable counts in roll tubes containing amylose azure rumen fluid agar (●——●) and by ^{3}H-thymidine uptake (▲——▲))


COUNTS PER MINUTE X 100
10
9
8
7
6
5
4
3
2
1
0
10
20
30
40
50
TIME, HOURS
10.0
9.5
9.0
8.5
LOG10 cfu/ml

These data also appear in Figure 6. By both methods of monitoring growth a generation time of 4 h and 20 min was observed. Also, the pH remained 6.8 throughout the 50 h of growth.

Amylase activity was monitored by the three amylase assays throughout the growth period. Averages of two experiments appear in Figure 7. The disc-plate assay clearly showed that maximum amylase production was attained when the stationary growth phase was attained. Amylase was produced throughout growth, increasing as the cell numbers increased. Results obtained using the reducing value and the blue value assays were not consistent with the data obtained by the disc plate assay. Problems with the three amylase assays will be examined in DISCUSSION.

Cells were removed from a 36 h culture by centrifugation, and the supernatant was analyzed for volatile compounds by gas-liquid chromatography. Strain 5LA produced 8.6 µM/ml ethanol, 20.1 µM/ml formate, 6.2 µM/ml acetate, and 6.8 µM/ml lactate. These figures were calculated by deducting levels of these compounds found in the uninoculated medium from the total amount after 36 h of growth.

As previously discussed in the LITERATURE REVIEW, induction and repression of microbial amylases have been studied by several researchers (Coleman and Elliott, 1962;

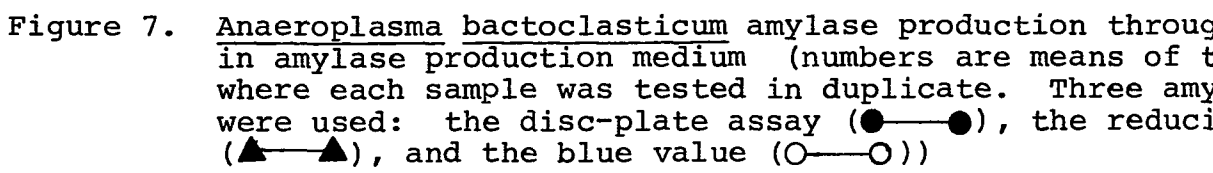
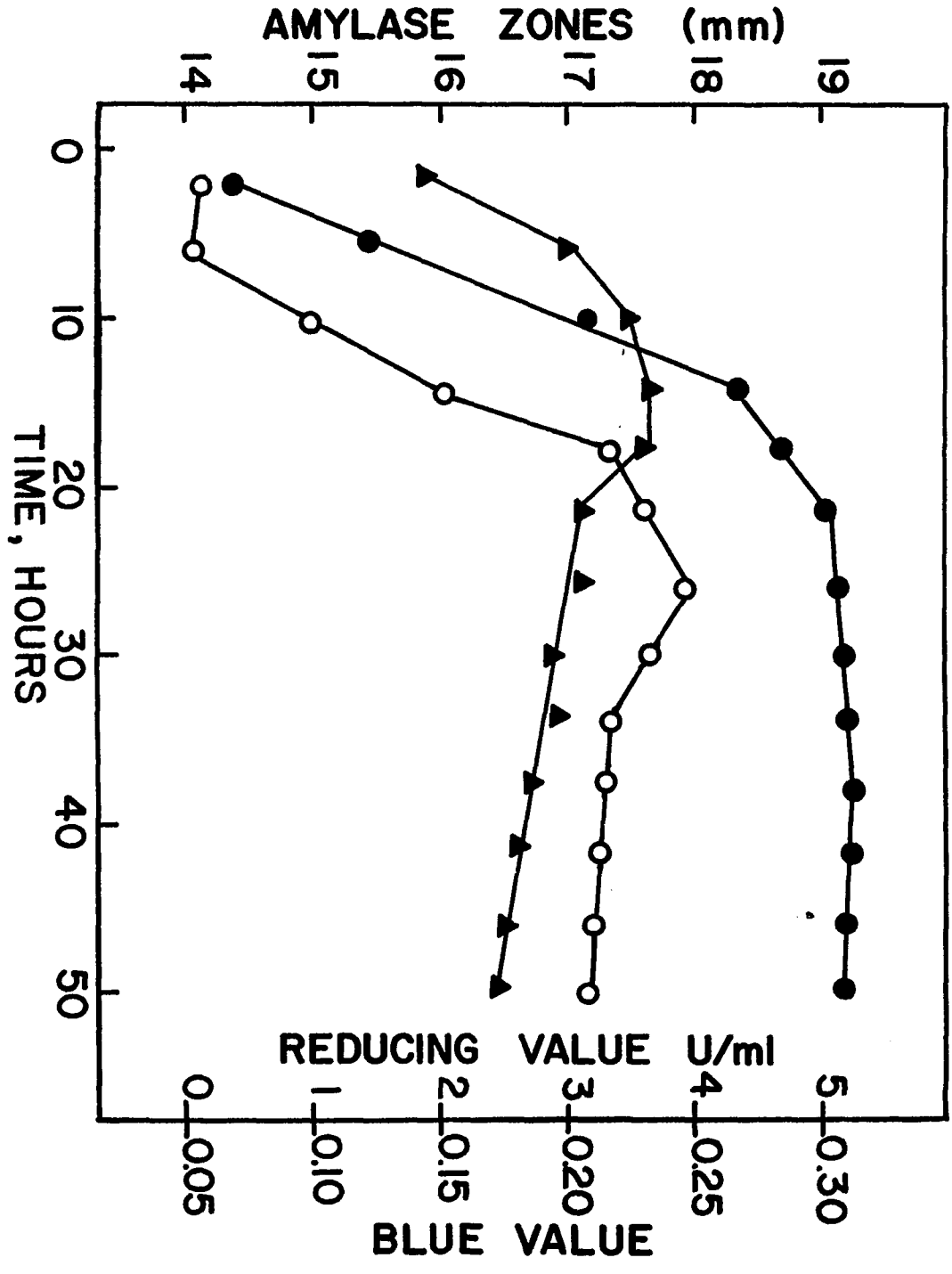


Figure 7. *Anaeroplasma bactoclasticum* amylase production throughout growth in amylase production medium (numbers are means of two experiments where each sample was tested in duplicate. Three amylase assays were used: the disc-plate assay (●——●), the reducing value (▲——▲), and the blue value (○——○))


AMYLASE ZONES (mm)
14
15
16
17
18
19
0
10
20
30
40
50
TIME, HOURS
REDUCING VALUE U/ml
0
1
2
3
4
5
0.05
0.10
0.15
0.20
0.25
0.30
BLUE VALUE

Griffin and Fogarty, 1973; Klein, 1963; Saito and Yamamoto, 1975; Tomomura et al., 1961). Research leading to the formulation of the production medium indicated that raw starchy materials resulted in maximum yields of Anaeroplasma amylase. The effects of a few carbohydrates (glucose, maltose, maltotriose and cellobiose) were studied further. These carbohydrates were examined in conjunction with maize starch or as sole carbon sources. The effect of the addition of 5 mM cyclic adenosine monophosphae (cAMP) to each of these media was also observed. These data are shown in Table 9.

The addition of these carbohydrates did not alter amylase production in the presence of maize starch. Amylase production was greatly decreased, however, when maize starch was not present. Amylase was produced when cellobiose was the only carbon source. This indicated that amylase production was not dependent on the presence of α1:4 glucosidic carbohydrates. Paper chromatography indicated that no detectable low molecular maltodextrins (glucose through maltoheptaose) were present in the uninoculated production medium. The addition of cAMP did not significantly affect amylase production.

Table 9. Percentage of amylase produced in production medium and production medium minus maize starch containing various carbon sources with or without the addition of cyclic AMP

Supplements	Percentage of amylase produced			
	Production medium		Production medium minus maize starch	
	No cAMP	Plus cAMP	No cAMP	Plus cAMP
Production medium control	100	97	-	-
Glucose (0.5%)	100	97	ND[a]	ND
Glucose (0.75%)	100	96	67	63
Maltose (0.75%)	100	96	57	54
Maltotriose (0.75%)	100	96	54	54
Cellobiose (0.75%)	100	96	59	57

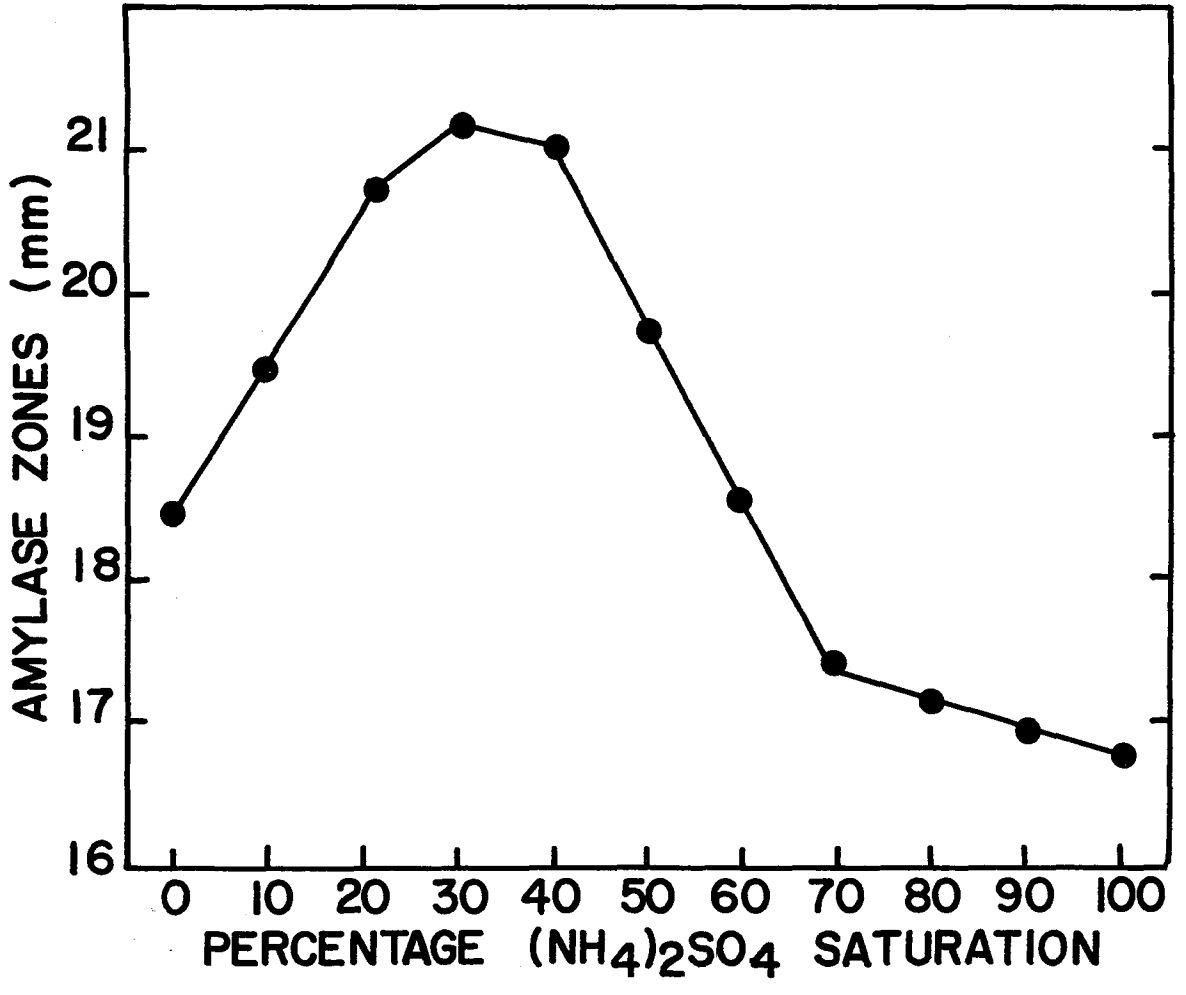
[a]ND means the experiment was not done.

Amylase Purification

Preliminary enzyme purification experiments were performed on the cell-free extract of 200-ml of a 24 h culture by using ammonium sulfate precipitation. Fractions were obtained by precipitating proteins to a 10% level of ammonium sulfate saturation. This fraction was removed by centrifugation, then a second fraction was precipitated by adding ammonium sulfate to a 20% saturation level. This stepwise procedure was followed until a 100% saturation fraction was obtained. Precipitates were resuspended and dialyzed for 48 h in 0.01 M PIPES plus 1 mM $CaCl_2$ at pH 6.0. Amylase concentrations, as detected by the disc-plate assay, are shown in Figure 8. Reducing value and blue value assays gave erratic, nonreproducible results. This was the first indication that these two assays were unreliable for this system (see DISCUSSION). Results of the disc-plate assay were reproducible. The $(NH_4)_2SO_4$ precipitation pattern was unique because the amylase activity increased from a zone size of 18.5 to 21.4 mm as the ammonium sulfate saturation was increased from 0% to 30%.

It was hypothesized that this ammonium sulfate precipitation curve may indicate an amylase inhibitor was precipitated out as the $(NH_4)_2SO_4$ concentration reached 30% saturation and removal of the inhibitor allowed greater amylase

Figure 8. Amylase activity in the supernatant fluid after the stepwise addition of ammonium sulfate (the disc-plate amylase assay was used for detection of amylase activity. The data pictured are the averages of three experiments)


AMYLASE ZONES (mm)
21
20
19
18
17
16
0 10 20 30 40 50 60 70 80 90 100
PERCENTAGE (NH4)2SO4 SATURATION

activity detection. This hypothesis was tested by adding the 0-10% and 10-20% fractions to the amylase fraction (20-50%). A number of concentration combinations were studied. The fractions were incubated at 42 C for 30 min prior to assaying for amylase activity. No inhibition effect was observed when these fractions were recombined with the amylase fraction.

The fractions of the ammonium sulfate precipitation included 0-10%, 10-20%, 20-50%, 50-70% and 70-100% precipitates. These fractions were dissolved in and dialyzed against 0.01 M PIPES containing 1 mM $CaCl_2$, pH 6.0. The bulk of the amylase was found in the 20-50% fraction giving zones 23.2 mm in diameter. This fraction was a light chocolate brown color. Only trace amounts of amylase (13.9 mm zone size) appeared in the fractions less than 20% ammonium sulfate saturation. No amylase activity was detected in the 50-70% or 70-100% fractions. The fractions were also checked for protease activity by using a disc-plate method; nutrient agar containing 1% dry skim milk was the assay medium. Protease activity was not detected when the cell-free extract was assayed whether or not 0.1% dithiothreitol was added to the extract before assay. However, the 70-100% ammonium sulfate fraction did possess protease activity (data not shown).

As discussed in MATERIALS AND METHODS, 9-1 cultures of

Anaeroplasma were grown to produce large quantities of this amylase. The culture was seeded with a 10% inoculum from a 25-h culture. During growth, culture purity was checked microscopically and by incubating trypticase soy agar streak plates aerobically. Amylase was harvested after 24 h of growth; a cell-free preparation was obtained by centrifugation at 16,300 x g for 30 min.

An ammonium sulfate precipitate of 20-50% saturation was suspended in and dialyzed against 0.01 M PIPES buffer containing 1 mM $CaCl_2$, pH 6.0. The fraction was dialyzed for 7 days, at which time SO_4^{-2} was no longer detected in the used buffer by precipitation with $BaCl_2$. After dialysis, the enzyme preparation was clarified by centrifugation for 20 min at 16,300 x g because a mucoid-like precipitate was present. The pellet was washed twice in 0.01 M PIPES $CaCl_2$ buffer. Most of the brown color remained in the supernatant. Both the supernatant and the pellet contained amylase. When 24 h digests of soluble starch and maltotriose by these two fractions were studied by paper chromatography the same action patterns were observed for both enzyme fractions. The enzyme produced large amounts of glucose and maltose as hydrolytic products. This indicated the same enzyme was present in both fractions.

Preliminary purification studies involved batch ion

exchange chromatography with DEAE-Sephadex A-50. The resin was treated by using a modification of the method of Baumstarck, Laffin and Burdawil (1964). Elution buffers were 0.01 M PIPES, pH 6.0, containing NaCl concentrations ranging from 0.1 to 0.5 M at 0.05 M increments. The results were surprising because amylase activity was eluted at all NaCl concentrations. Additional amylase activity was eluted when the molarity was increased to 1 M but not when the molarity was increased further to 2 M. Active amylase was not eluted when the pellet fraction was treated by batch ion exchange chromatography. Attempts to remove absorbed amylase by increasing the salt concentration to 3 M, rinsing with buffer not containing NaCl, or rinsing with buffer containing either 1, 5 or 10% ethylene glycol were all unsuccessful. Ethylene glycol was tested because it was hypothesized that the enzyme was possibly bound by hydrophobic bonds. Because work with the viscous pellet fraction would have been extremely difficult, further studies involved only the supernatant.

Although batch ion exchange with DEAE-Sephadex A-50 was unsuccessful in removing the amylase activity from the complex precipitate, this procedure was effective in decolorizing the enzyme fraction. Thus, this step was incorporated in the purification procedure because decolorization experiments with activated charcoal were unsuccessful.

When a 0-70% ammonium sulfate precipitate was tested, however, the fraction was not completely decolorized by batch ion exchange chromatography. The procedure developed for the partial purification of Anaeroplasma bactoclasticum 5LA extracellular amylase appears in Figure 9.

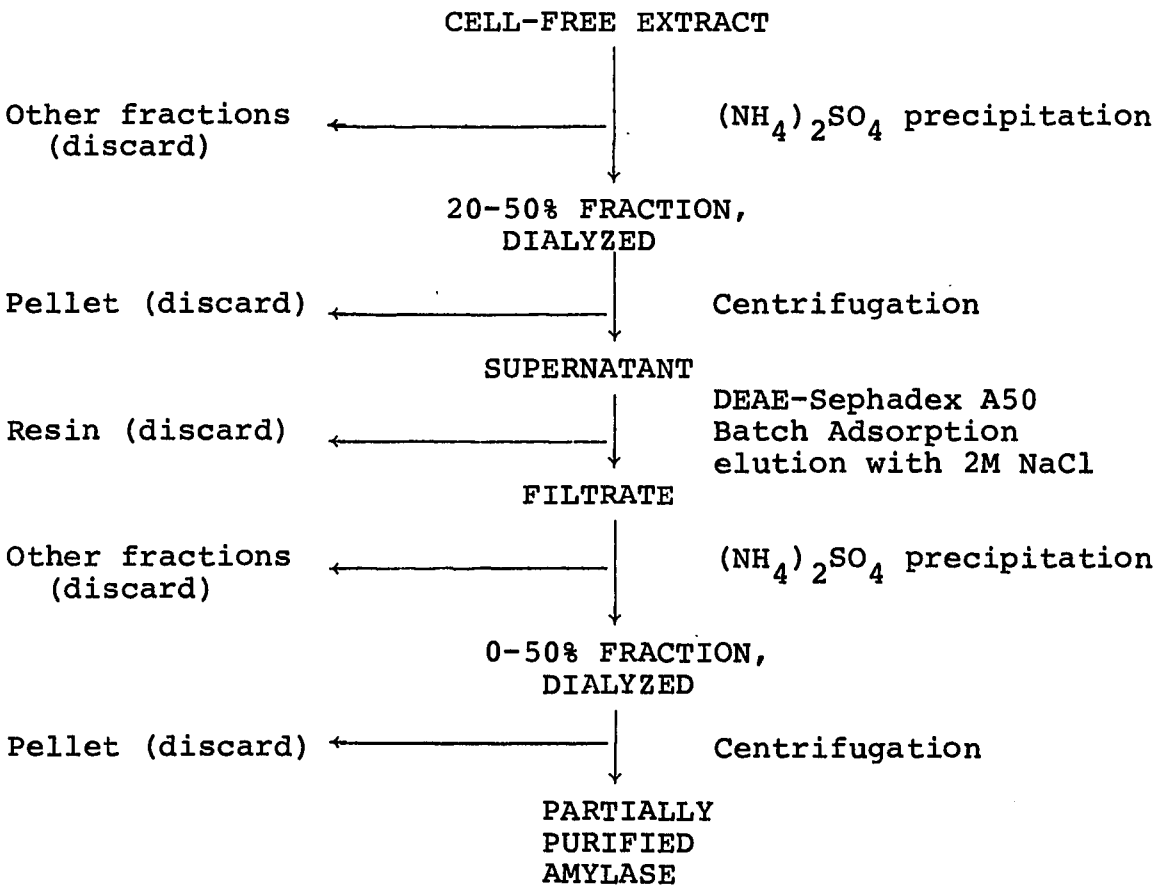


Figure 9. Anaeroplasma bactoclasticum amylase purification

The partially purified amylase was applied to a DEAE-Sephadex A-50 (Pharmacia, Inc., Piscataway, NJ) column for column chromatography. A gradient of 0.1 to 0.5 M NaCl in 0.01 M PIPES at pH 6.0 was used as the elution buffer. Only trace amounts of amylase were eluted. There was also no specific elution peak; trace amounts of amylase were eluted throughout the NaCl gradient. Therefore, DEAE-sephadex column chromatography was not a satisfactory means of Anaeroplasma amylase purification.

Protein separation was then attempted by using Sephadex G-200 (Pharmacia, Inc., Piscataway, NJ) column chromatography. The results are shown in Figure 10. Amylase was eluted over an elution volume of 280 ml. Distinct peaks were not evident. Because the bulk of the amylase activity was in a region of high protein concentration, further attempts to find a better method of protein purification were made. Column chromatography with Biogel A 0.5 m (Bio Rad Laboratories, Richmond, CA) was tested. As shown in Figure 11, no separation was attained with Biogel A 0.5 m.

Consequently, a number of ion-exchange media were tested for their ability to reversibly absorb the Anaeroplasma bactoclasticum 5LA extracellular amylase. The ion exchangers, chosen for their various functional groups, were prepared as described by the manufacturers. Excess buffer was removed

Figure 10. Elution pattern of Anaeroplasma bactoclasticum partially purified amylase from a Sephadex G-200 column (the column was 2.5 x 87 cm with a void volume of 116 ml and a flow rate of 12 ml/h. 5 ml fractions were taken. The end of the void volume was denoted as 0 ml. Protein (⊢——⊣) was monitored by absorbance at 280 nm and amylase activity (⊢– – –⊣) was detected by the disc-plate assay)

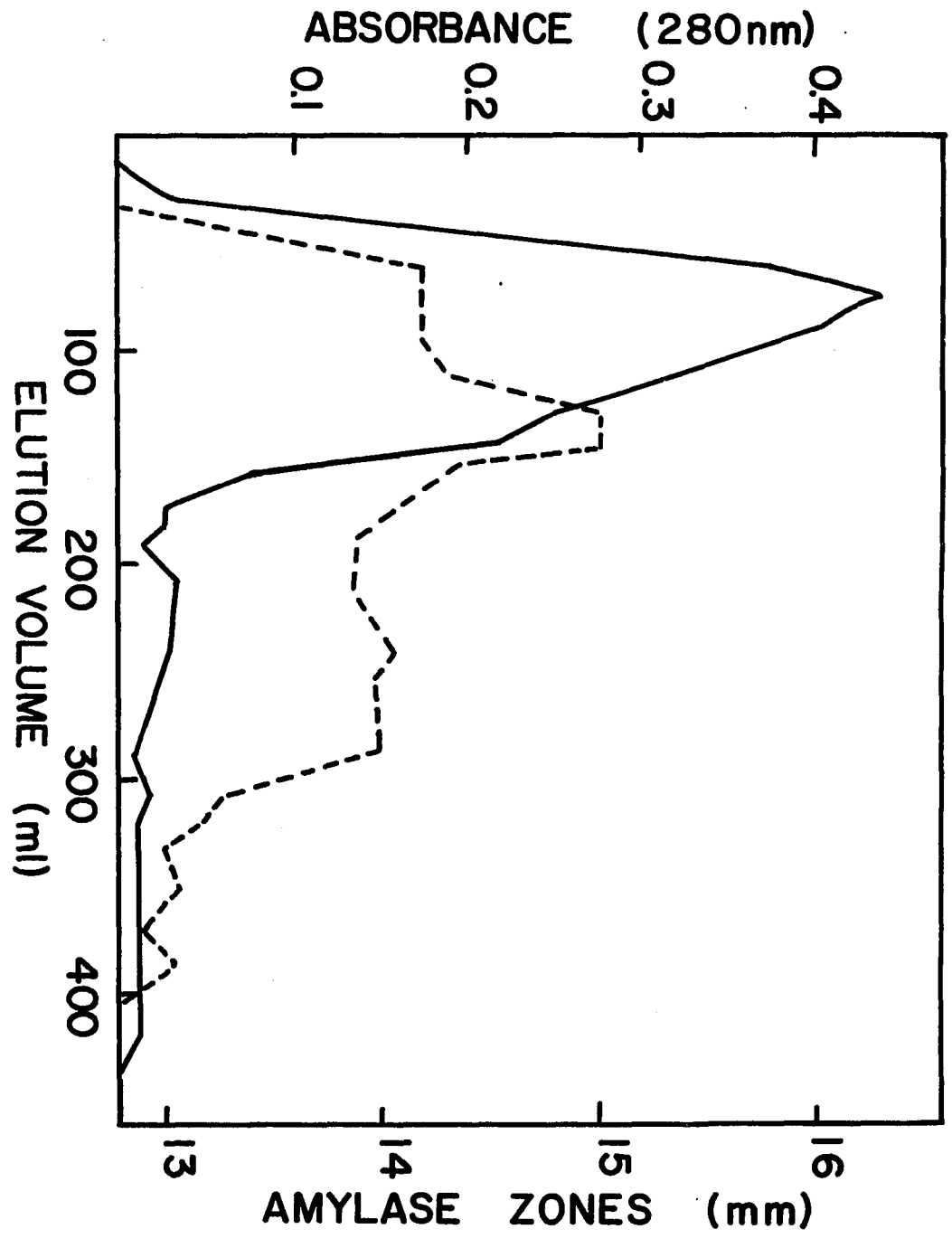
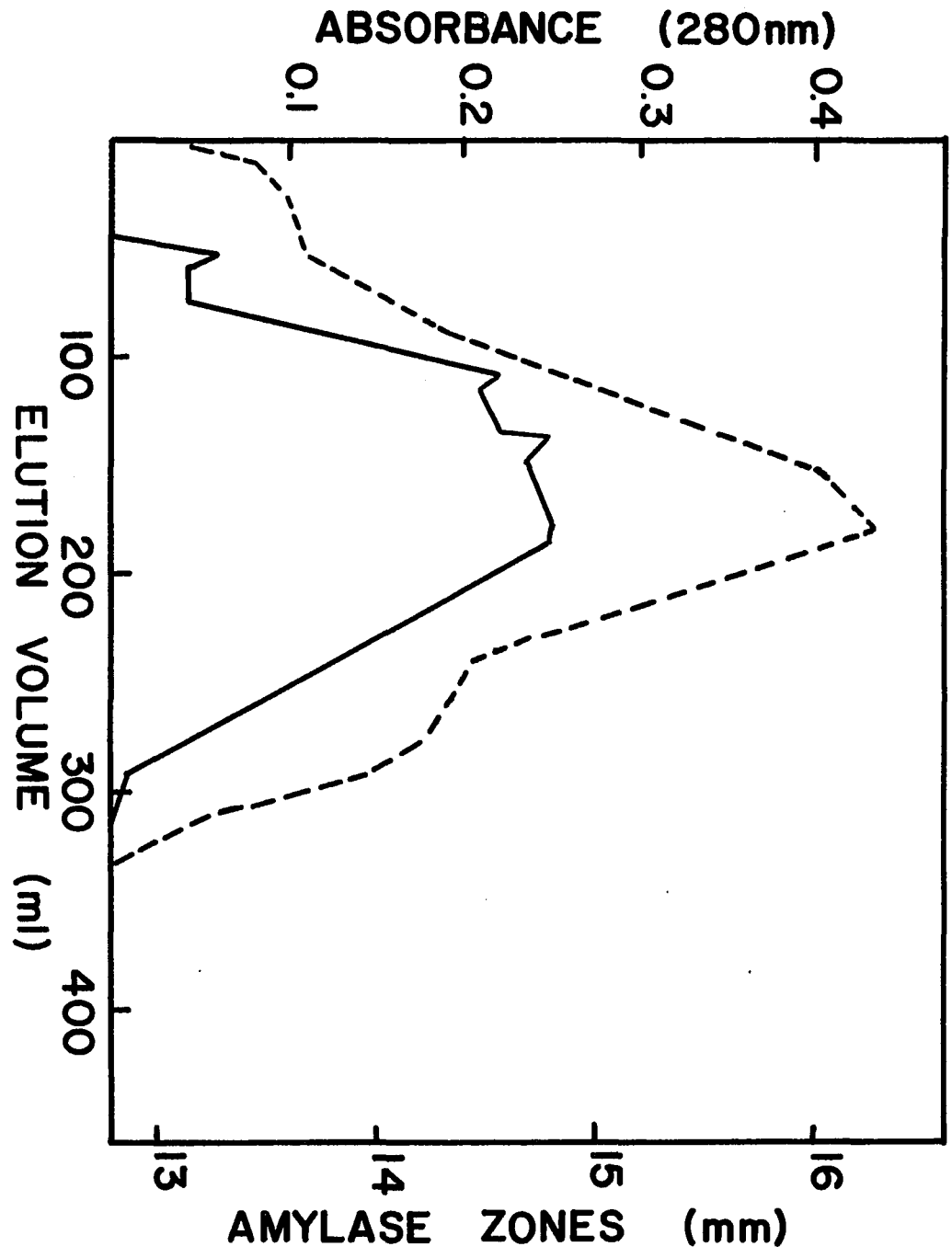

ABSORBANCE (280nm)
0.1
0.2
0.3
0.4
ELUTION VOLUME (ml)
100
200
300
400
13
14
15
16
AMYLASE ZONES (mm)

Figure 11. Elution pattern of Anaeroplasma bactoclasticum partially purified amylase from a Biogel A 0.5 m column (the column was 2.5 x 91 cm with a void volume of 135 ml and a flow rate of 14 ml/h. 5 ml fractions were taken. The end of the void volume was denoted as 0 ml. Protein (⟵⟶) was monitored by absorbance at 280 nm and amylase activity (⟵----⟶) was detected by the disc-plate assay)


ABSORBANCE (280nm)
0.1
0.2
0.3
0.4
100
200
300
400
ELUTION VOLUME (ml)
13
14
15
16
AMYLASE ZONES (mm)

from each ion-exchange medium, contained in a 16 x 150 mm test tube, and 1 ml of partially purified enzyme plus 1 ml 0.01 M PIPES containing 1 mM $CaCl_2$ were added, then mixed. The enzyme was allowed to absorb overnight at 4 C. The supernatant was assayed by the disc-plate method to detect unabsorbed amylase. The amylase was eluted from the resins by using 0.01 M PIPES plus 1 M NaCl. After mixing, the enzyme was allowed to elute overnight at 4 C. The supernatant was assayed again after elution. The results are shown in Table 10. DEAE-Sepharose CL-6B (Pharmacia, Inc., Uppsala, Sweden) was chosen for further ion exchange experiments. Figure 12 shows that no protein separation was attained by this method.

Problems with the amylase assays resulting in inaccurate calculations of specific activity and percent yield (see DISCUSSION) prompted a reexamination of the purification schema.

To examine the proteins present in the original, untreated cell-free extract, a sample was studied by Sephadex G-200 column chromatography. These data are illustrated in Figure 13. The proteins of clarified rumen fluid were also determined by Sephadex G-200 column chromatography. The large protein peak from 310 to 600 ml elution volume in the elution pattern of the cell-free extract, Figure 13,

Table 10. Absorption and elution of Anaeroplasma bactoclasticum partially purified amylase from various ion exchangers

Ion exchanger and manufacturer	Percentage			
	Absorbed	Not absorbed	Eluted[a]	Not absorbed plus eluted
ANION EXCHANGERS				
DEAE TLC cellulose[b]	62	38	71	109
CELU ION DEAE[b]	77	23	59	82
Selectacel DEAE-standard[c]	36	64	65	129
Selectacel DEAE-type 20[c]	59	41	64	105
Selectacel DEAE-type 40[c]	56	44	57	101
DEAE Sephadex A50[d]	100	0	23	23
DEAE Sepharose CL-6B[d]	19	81	50	131
CELU ION TEAE[b]	79	21	60	81
Cellex-T[e]	68	32	52	84
CELU ION AE[b]	85	15	64	79
Cellex-AE[f]	34	66	61	127
CELU ION ECTEOLA[b]	87	13	56	69
Cellex-E[e]	18	82	35	117
Dowex 1-X4[f]	31	69	27	96
Dowex 1-X4[b,e]	48	52	47	99
Dowex 2-X[e]	19	81	31	112
Dowex 2-X[e,g]	36	64	32	96

[a] Eluted with 0.01 M PIPES containing 1 M NaCl, pH 6.0.

[b] Nutritional Biochemical Corp., Cleveland, OH.

[c] Carl Schleicher and Schuell Co., Keene, NH.

[d] Pharmacia, Inc., Piscataway, NJ.

[e] Bio Rad Laboratories, Richmond, CA.

[f] J. T. Baker Chemical Co., Phillipsburg, NJ.

[g] Indicates test used 1.0 g of ion exchanger. All others used 0.2 g of exchanger.

Table 10 (Continued)

Ion exchanger and manufacturer	Percentage			
	Absorbed	Not absorbed	Eluted[a]	Not absorbed plus eluted
ANION EXCHANGERS (Cont.)				
Bio-Rex[e]	57	43	13	56
Bio-Rex[e,g]	82	18	11	29
AG-3-X4[e]	36	64	4	68
AG-3-X4[e,g]	85	15	4	19
CATION EXCHANGERS				
CELU ION CM[b]	79	21	0	21
CELU ION P[b]	56	44	23	67
CELU ION SE[b]	18	82	37	119
Cellex-SE[e]	29	71	16	87
Amberlite MB-3[h]	16	84	8	92
Amberlite MB-3[g,h]	100	0	0	0
Amberlite IRC-50CP[h]	37	63	3	66
Amberlite IRC-50CP[g,h]	56	44	11	55

[h]Mallinkrodt Chemical Works, St. Louis, MO.

Figure 12. Elution pattern of Anaeroplasma bactoclasticum partially purified amylase from a DEAE-Sepharose CL-6B column (the column was 2.5 x 35 cm with a void volume of 108 ml and a flow rate of 10 ml/h. 5 ml fractions were taken. The end of the void volume is denoted as 0 ml. Protein (⟵——⟶) was monitored by absorbance at 280 nm and amylase activity (⟵——⟶) was detected by the disc-plate assay. Approximate molarity (⟵·—·⟶) is also indicated)

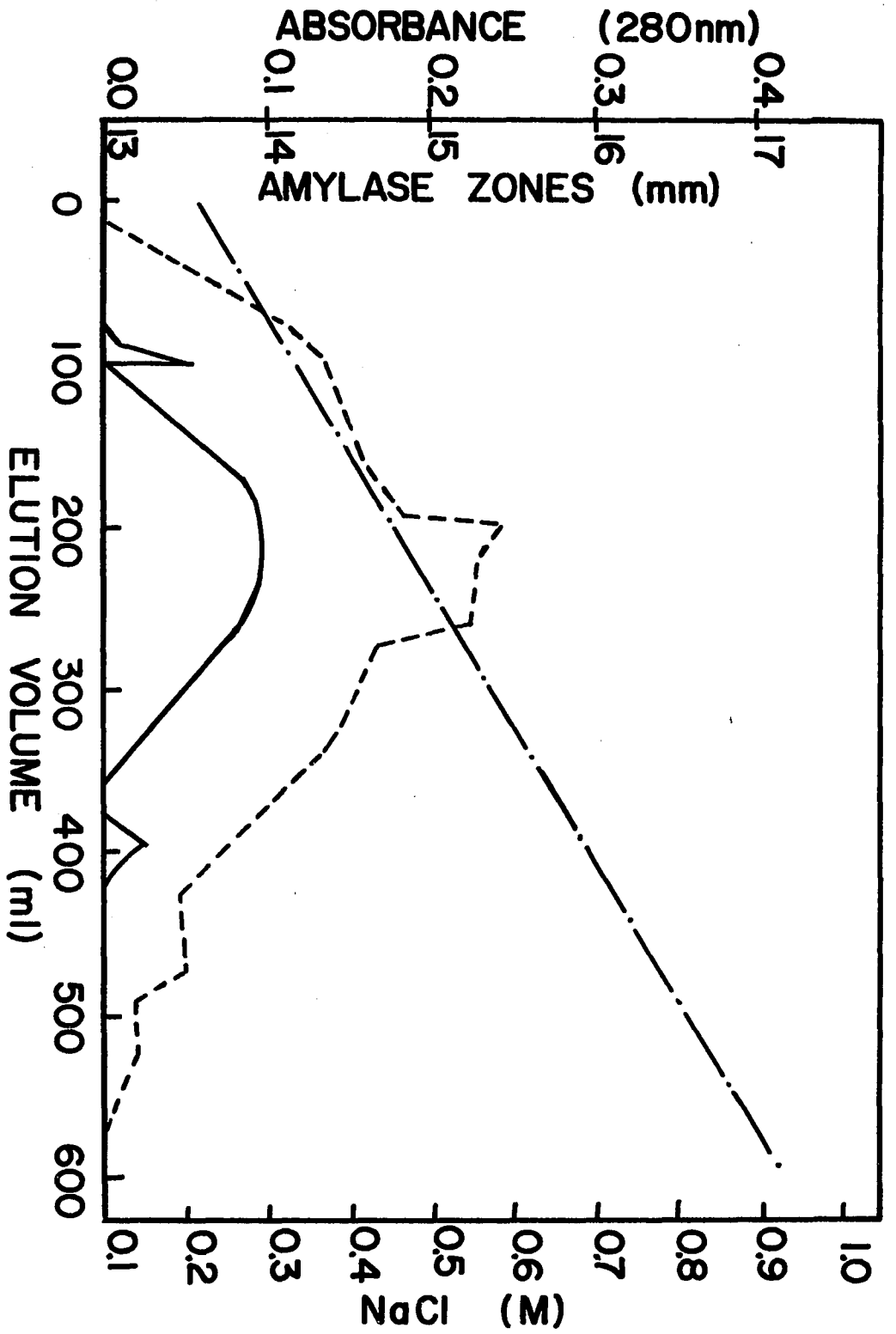
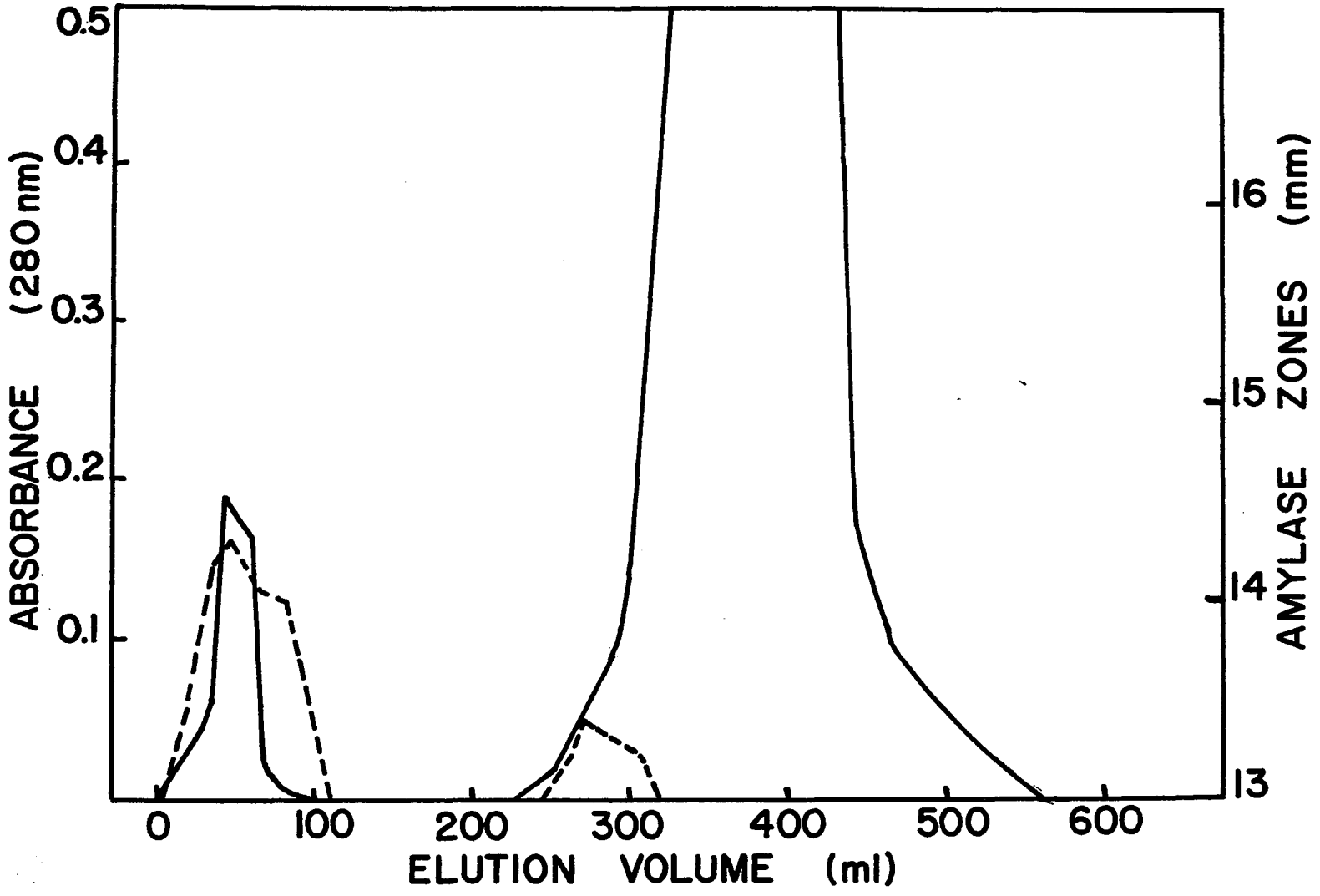

ABSORBANCE (280nm)
0.0
0.1
0.2
0.3
0.4
I3
I4
I5
I6
I7
AMYLASE ZONES (mm)
0
100
200
300
400
500
600
ELUTION VOLUME (ml)
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
NaCl (M)

Figure 13. Elution pattern of Anaeroplasma bactoclasticum cell-free extract from a Sephadex G-200 column (the column was 2.5 x 87 cm with a void volume of 116 ml and a flow rate of 12 ml/h. The end of the void volume is denoted as 0 ml. 5 ml fractions were taken. Protein (⟷) was monitored by absorbance at 280 nm and amylase activity (⟷) was detected by the disc-plate assay)


0.5
0.4
0.3
0.2
0.1
ABSORBANCE (280 nm)
16
15
14
13
AMYLASE ZONES (mm)
0
100
200
300
400
500
600
ELUTION VOLUME (ml)

corresponds with proteins found in clarified rumen fluid. The proteins eluted with amylase activity in Figure 13 were not present when clarified rumen fluid was examined.

None of the above chromatographic techniques gave satisfactory separation of Anaeroplasma bactoclasticum 5LA extracellular amylase from other proteins present in the partially purified amylase sample. Consequently, attempts at further purification had to be abandoned insofar as the present study is concerned and enzyme characterization experiments were performed by using the preparation of partially purified amylase.

Characterization of Partially Purified Amylase

Experiments leading to the parameters of the disc-plate assay implied that the optimum pH of the cell-free amylase was 6.0, Figure 3. This was verified by several experiments, using the partially purified enzyme. The effect of pH was tested by two means: (i) the pH of the disc-plate assay agar was adjusted and (ii) the enzyme was incubated for 24 h at 42 C in a test buffer prior to assaying by the disc-plate method. In the latter experiment the following buffers were used: 0.01 M acetic acid-sodium acetate (pH 3.5 to 5.5), 0.01 M PIPES (pH 4.5 to 8.5) and 0.01 M borax-KH_2PO_4 (pH 6.0 to 9.0). Each buffer

contained 1 mM $CaCl_2$. These data appear in Figure 14.

The temperature optimum of the partially purified amylase was 42-45 C. This was determined by incubating assay plates at various temperatures for the assay incubation period. Although temperature effects the diffusion rate as well as enzyme activity, this curve appeared to indicate the temperature optimum. The results appear in Figure 15. Temperature sensitivity was tested by incubating the enzyme in 0.01 M PIPES containing 1 mM $CaCl_2$ at pH 6.0 for 0, 1, 10, 30, 60, and 90 min at various temperatures before assaying. The results are shown in Figure 16. The enzyme was stable at 4 and 25 C over the 90 min period. At 42 C, an initial decrease in activity to 80% was observed at 10 min; however, for the remaining 80 min the enzyme was stable at 80% relative activity. Decreasing amylase stability was observed as the temperature increased. At 62 C, 90% of the enzyme was inactivated within 10 min, and no activity could be detected after 60 min.

The action of Anaeroplasma amylase on soluble starch was studied by paper chromatography. As shown in Figure 17 as the time of enzyme-substrate interaction increased large amounts of glucose and maltose were produced. Because other microbial amylases generally produce only small amounts of glucose, if at all, further investigations of

Figure 14. Anaeroplasma bactoclasticum amylase activity as a function of pH (graph A represents enzyme activity detected by the disc-plate assay where the pH of the assay medium was adjusted. Graph B represents enzyme activity detected by the usual disc-plate assay. The enzyme was previously incubated for 15 min at 42 C in a test buffer adjusted to the indicated pH. The buffers used are represented as follows: 0.01 M PIPES (●——●), 0.01 M acetic acid-sodium acetate (▲——▲) and 0.01 M borax-KH_2PO_4 (○——○))

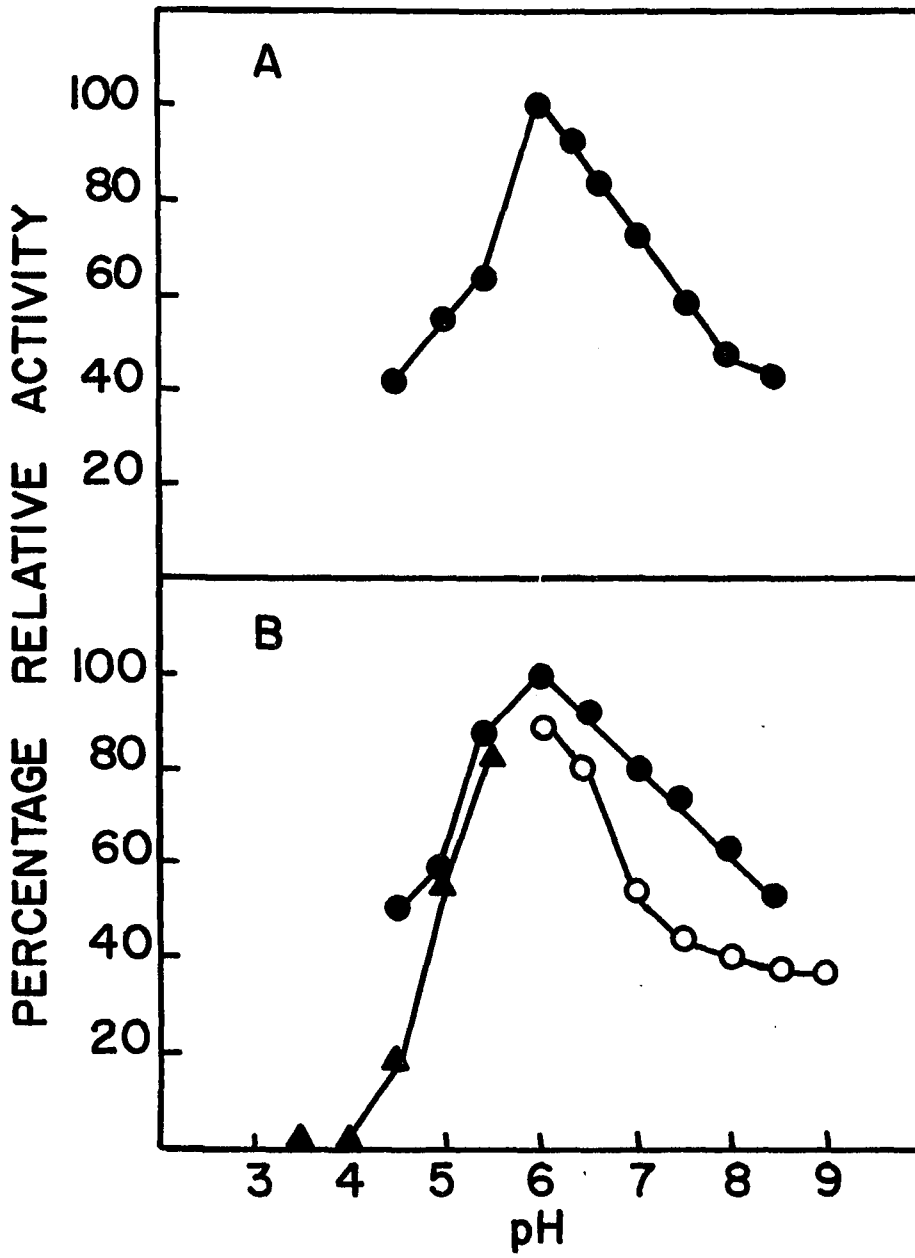

A
B
100
80
60
40
20
PERCENTAGE RELATIVE ACTIVITY
3
4
5
6
7
8
9
pH

Figure 15. Anaeroplasma bactoclasticum amylase activity as a function of temperature (amylase activity was detected by the disc-plate assay. Plates were incubated at the indicated temperature)

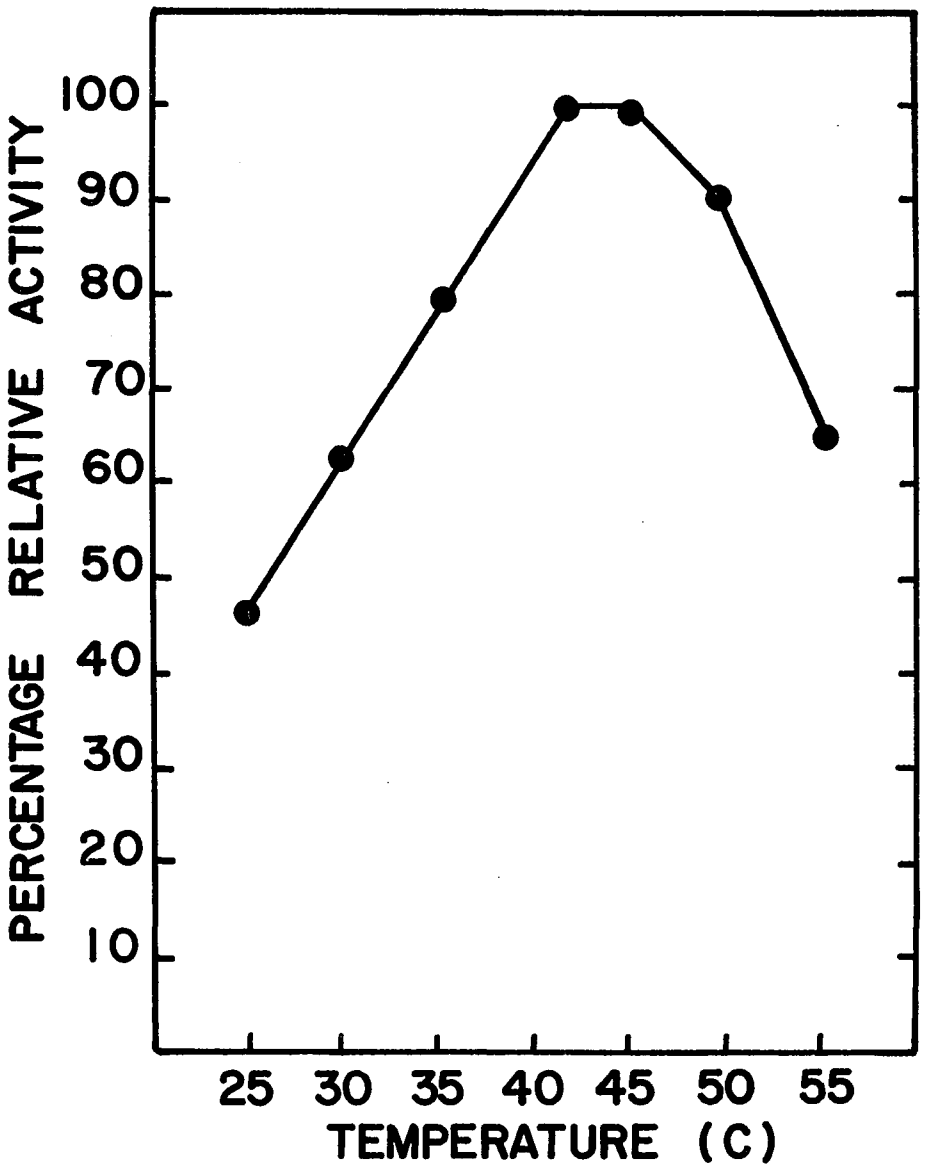

PERCENTAGE RELATIVE ACTIVITY
100
90
80
70
60
50
40
30
20
10
25 30 35 40 45 50 55
TEMPERATURE (C)

Figure 16. Temperature sensitivity of Anaeroplasma bactoclasticum (the enzyme was incubated in 0.01 M PIPES containing 1 mM $CaCl_2$, pH 6.0 for the indicated time periods at the indicated temperature; the enzyme was then assayed by the disc-plate method)

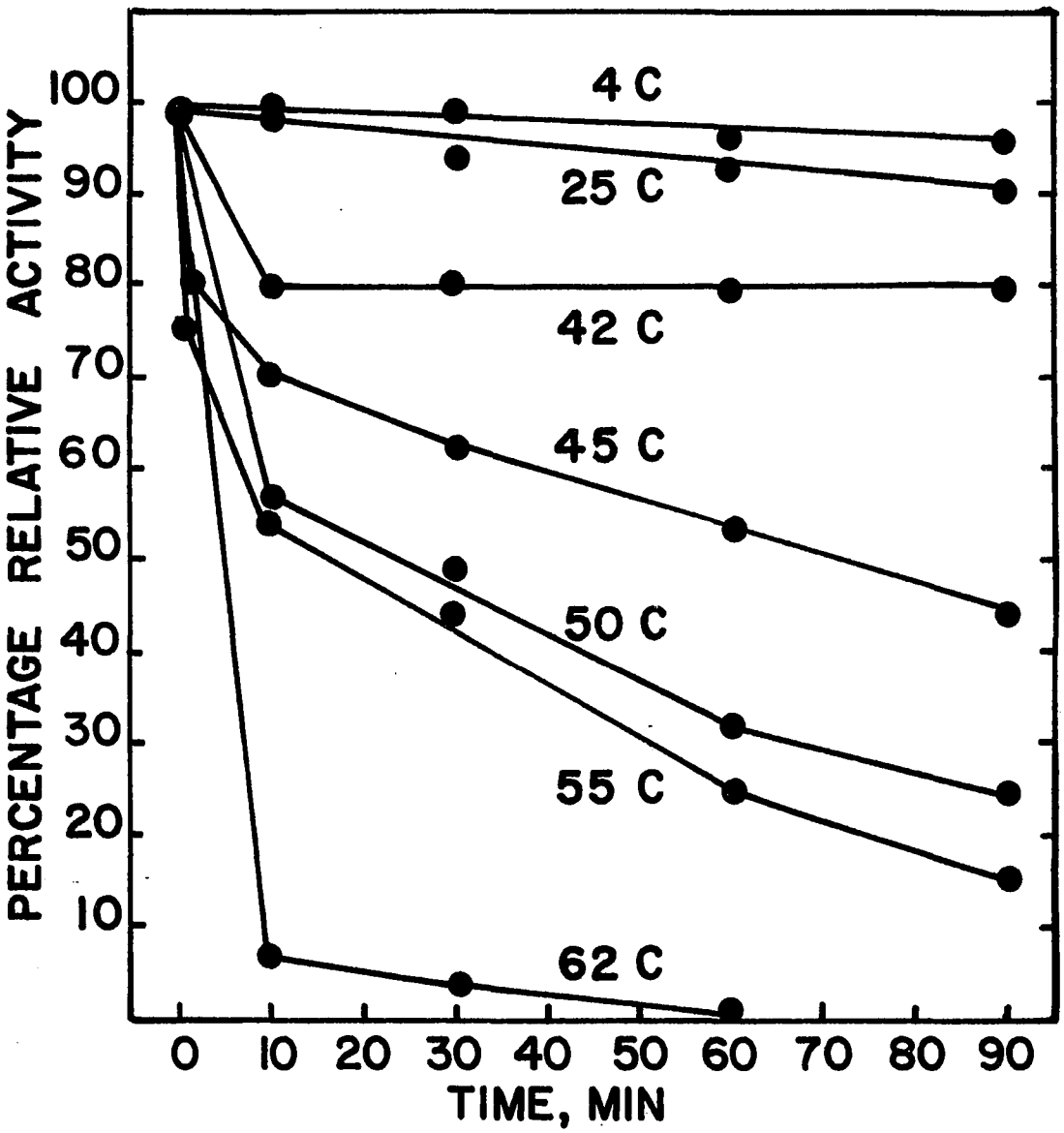
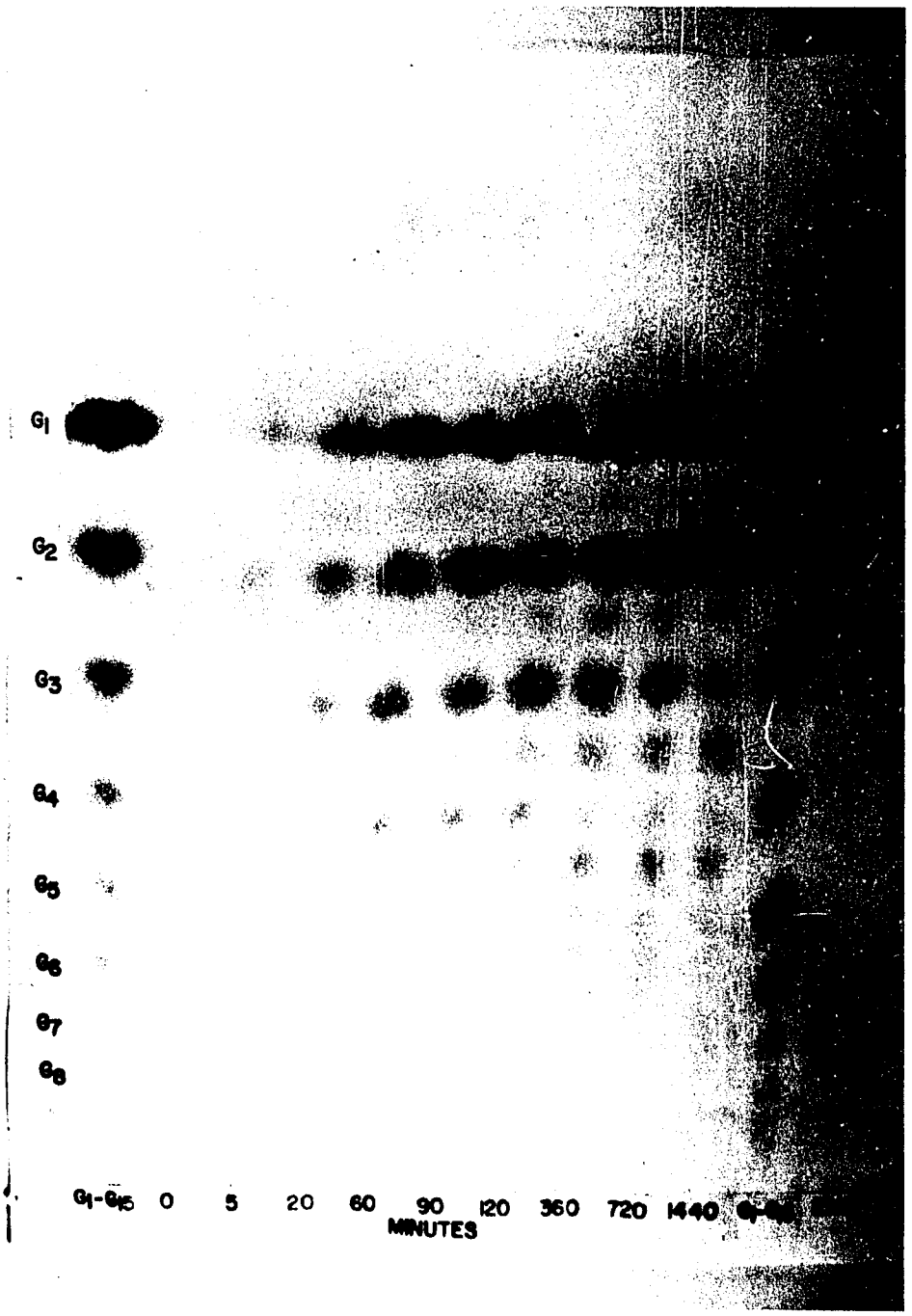

PERCENTAGE RELATIVE ACTIVITY
100
90
80
70
60
50
40
30
20
10
4 C
25 C
42 C
45 C
50 C
55 C
62 C
0
10
20
30
40
50
60
70
80
90
TIME, MIN

Figure 17. Chromatographic analysis of Anaeroplasma bactoclasticum partially purified amylase action on soluble starch (reaction mixtures combined 5 mg/ml soluble starch and 0.1 ml/ml enzyme. The enzyme preparation produced amylase zone sizes of approximately 19.0 mm on the disc-plate assay)


G1
G2
G3
G4
G5
G6
G7
G8
G1-G15
0
5
20
60
90
120
360
720
1440
MINUTES

substrate specificity and digestion were made.

The following substrates were examined by making paper chromatographs of enzyme-substrate reaction mixtures: maltose, cellobiose, iso-maltose, maltotriose, iso-maltotriose, maltotetraose, maltrin-10, G_{40}, amylose, amylopectin, potato starch granules and maize starch granules. The chromatograms of iso-maltose and iso-maltotriose confirmed that no activity was present that could cleave α1:6 glucosidic bonds. There was also no activity on cellobiose, demonstrating that the enzyme did not cleave β-1:4-glucosidic bonds. Activity was slower on the higher molecular weight carbohydrates. This was observed when the substrates were maize starch granules, Figure 18; amylopectin, Figure 19; amylose (not solubilized), Figure 20; and G_{40}, Figure 21. By 24 h only a trace amount of glucose, maltose and maltotriose had been hydrolyzed from potato starch granules. Maltrin-10, a maltodextrin mixture with an average degree of polymerization of 10, contained trace amounts of low molecular weight maltodextrins (G_2 through G_7). Thus, evidence from this chromatogram was not conclusive. Figure 22 is a chromatogram of amylase activity on maltotetraose, G_4. By 30 min some activity was observed; maltose (G_2) and maltotriose (G_3) were produced in trace amounts. At 90 min trace amounts

Figure 18. Chromatographic analysis of Anaeroplasma bactoclasticum partially purified amylase action on maize starch granules (reaction mixtures contained 5 mg/ml maize starch granules and 0.1 ml/ml enzyme. The amylase preparation produced amylase zone sizes of approximately 19.0 mm on the disc-plate assay)

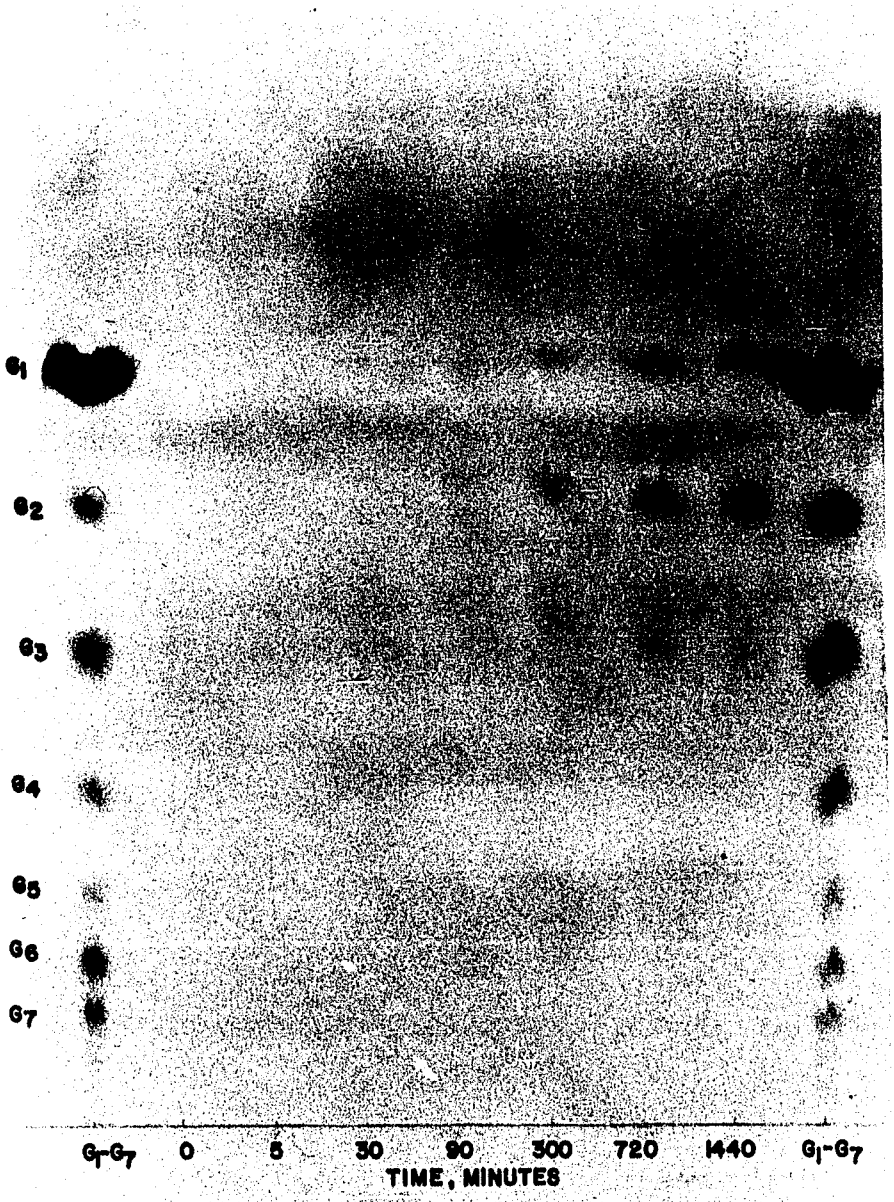

G1
G2
G3
G4
G5
G6
G7
G1-G7
0
5
30
90
300
720
1440
G1-G7
TIME, MINUTES

Figure 19. Chromatographic analysis of Anaeroplasma bactoclasticum partially purified amylase action on amylopectin (reaction mixtures contained 5 mg/ml amylopectin and 0.1 ml/ml enzyme. The enzyme preparation produced amylase zone sizes of approximately 19.0 mm on the disc-plate assay)

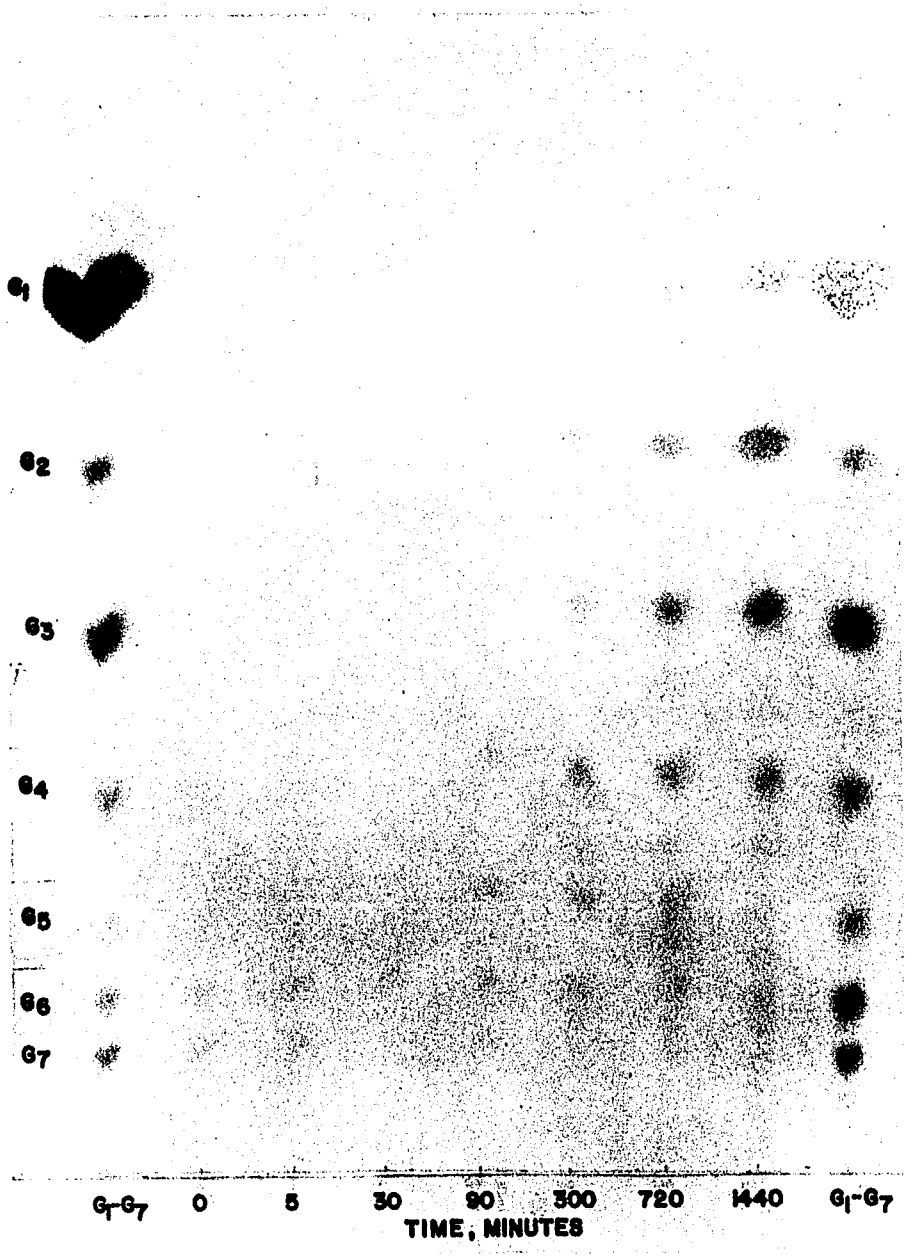

G1
G2
G3
G4
G5
G6
G7
G1-G7
0
5
30
90
300
720
1440
G1-G7
TIME, MINUTES

Figure 20. Chromatographic analysis of Anaeroplasma bactoclasticum partially purified amylase action on amylose (not solubilized) (reaction mixtures contained 5 mg/ml amylose and 0.1 ml/ml enzyme. The enzyme preparation produced amylase zone sizes of approximately 19.0 mm on the disc-plate assay)

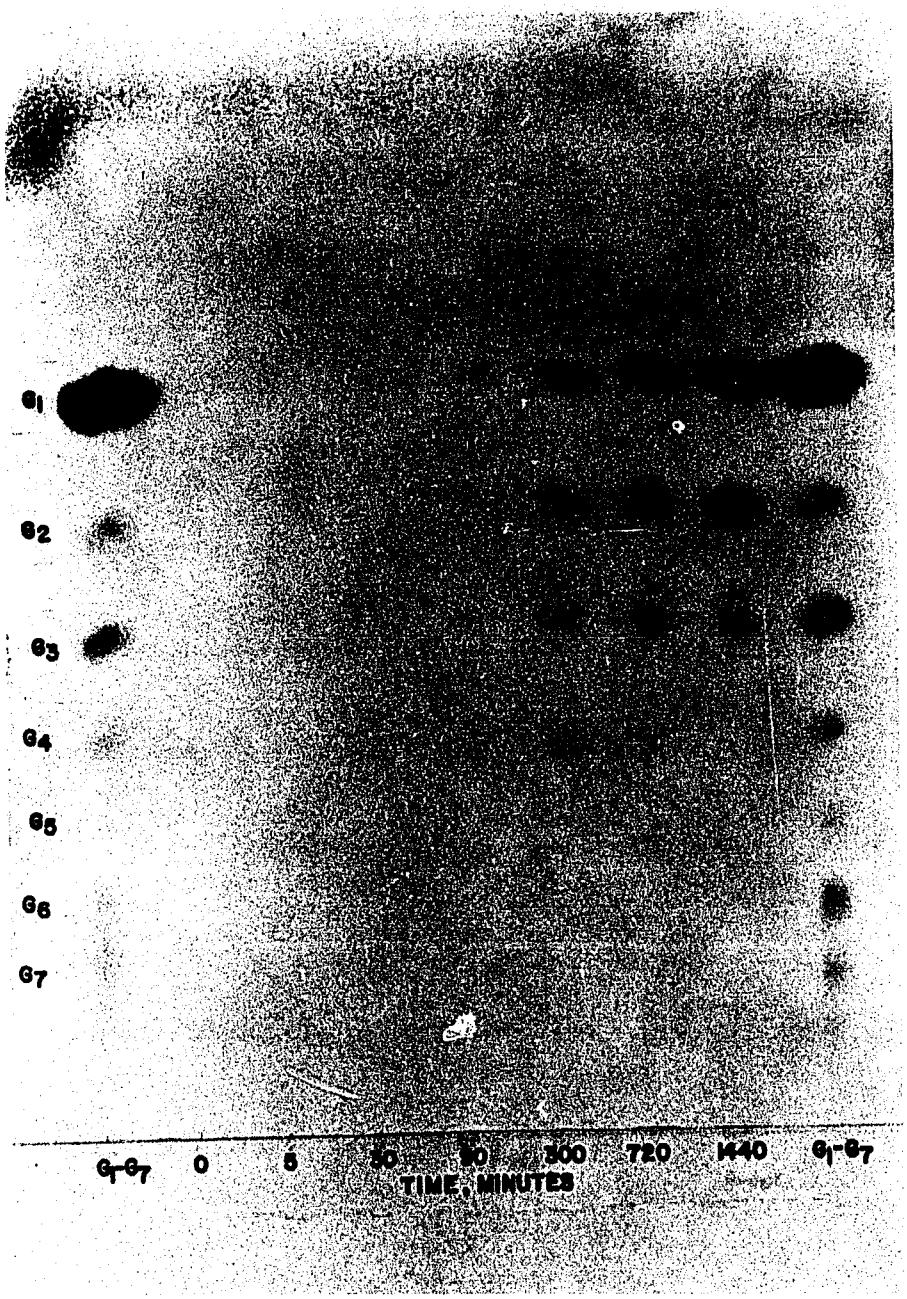

G1
G2
G3
G4
G5
G6
G7
G1-G7
0
5
30
90
300
720
1440
G1-G7
TIME, MINUTES

Figure 21. Chromatographic analysis of Anaeroplasma bactoclasticum partially purified amylase action on G_{40} (reaction mixtures contained 5 mg/ml G_{40} and 0.1 ml/ml enzyme. The enzyme preparation produced amylase zone sizes of approximately 19.0 mm on the disc-plate assay)

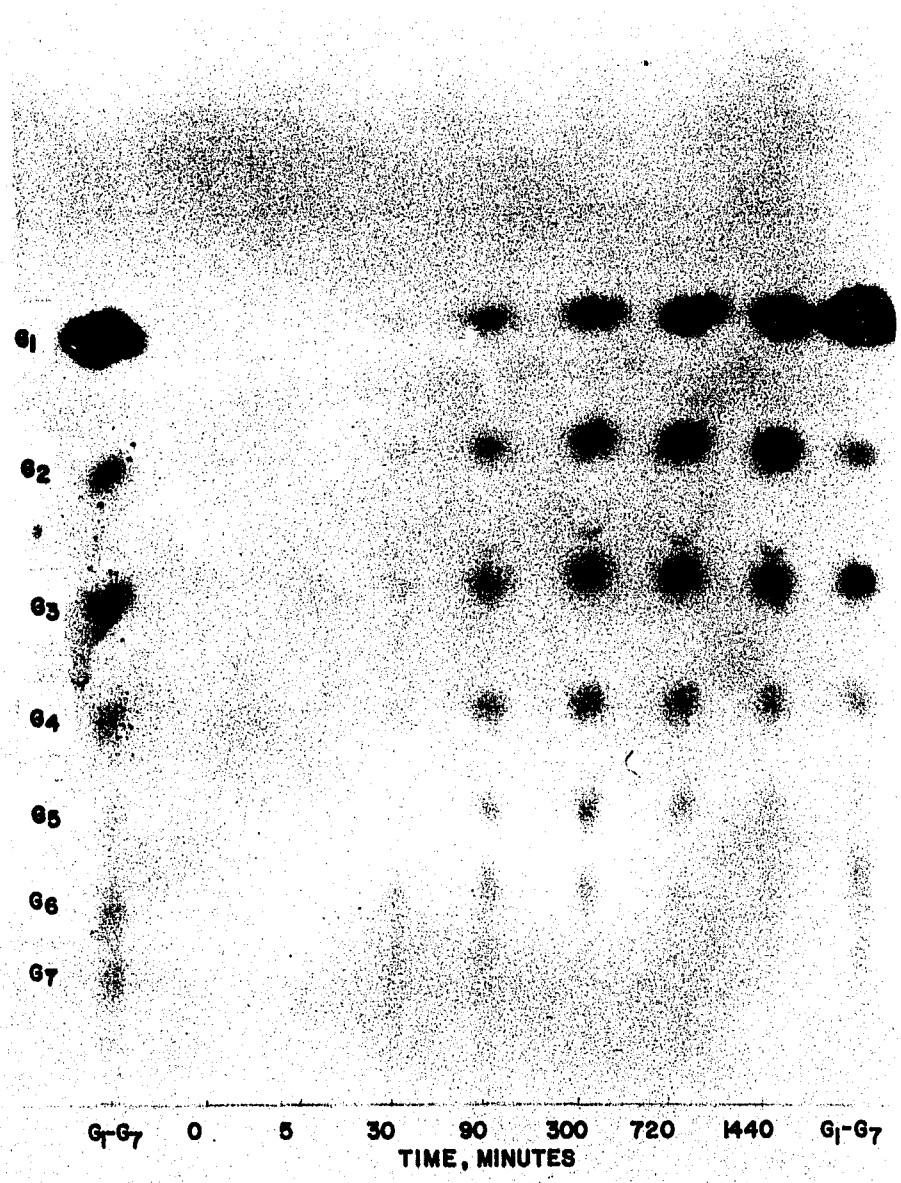
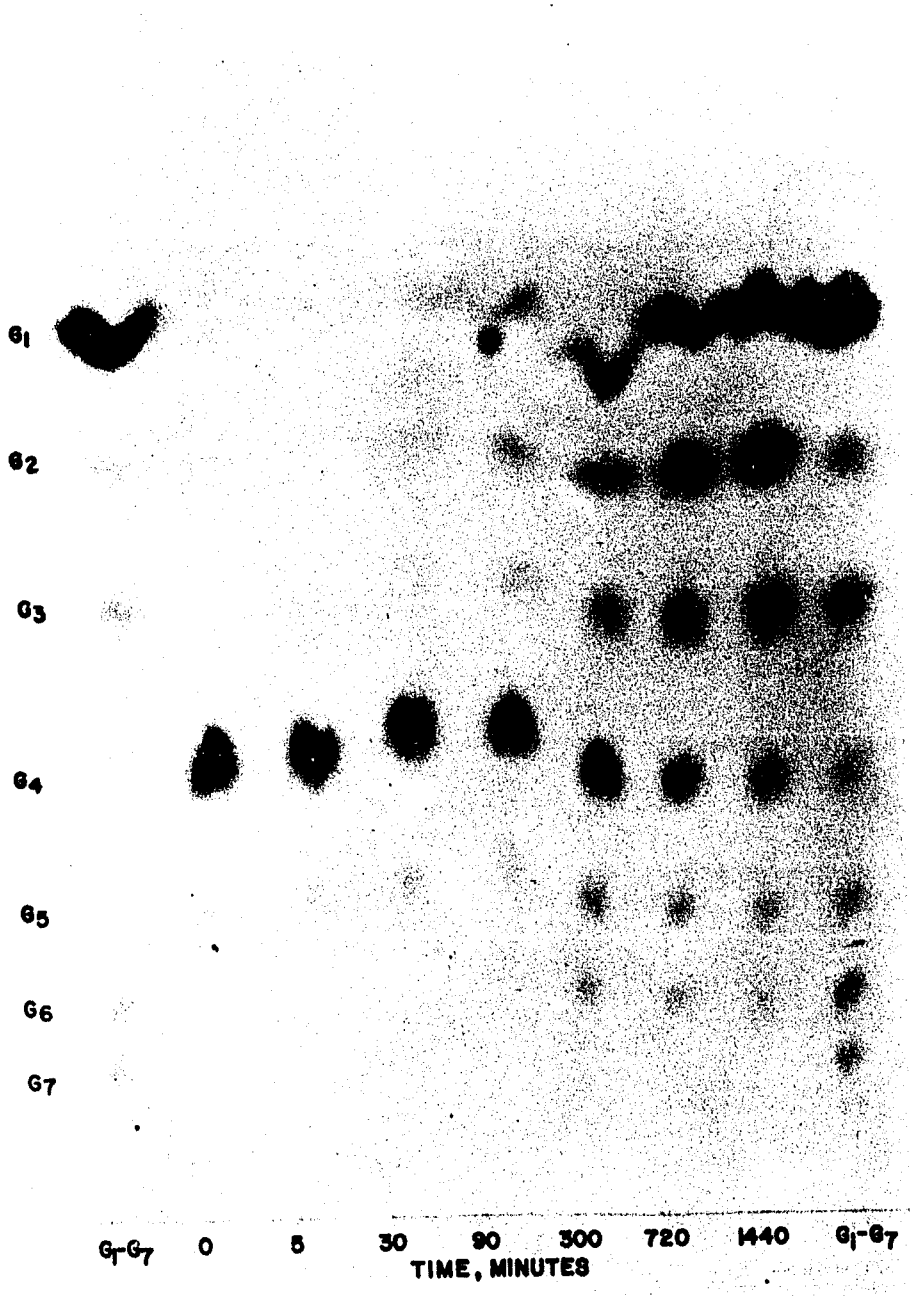

G1
G2
G3
G4
G5
G6
G7
G1-G7
0
5
30
90
300
720
1440
G1-G7
TIME, MINUTES

Figure 22. Chromatographic analysis of Anaeroplasma bactoclasticum partially purified amylase action on maltotetraose (reaction mixtures contained 5 mg/ml maltotetraose and 0.1 ml/ml enzyme. The enzyme preparation produced amylase zone sizes of approximately 19.0 mm on the disc-plate assay)


G1
G2
G3
G4
G5
G6
G7
G1-G7
0
5
30
90
300
720
1440
G1-G7
TIME, MINUTES

of glucose (G_1), were also observed. Maltotriose, Figure 23, was one of the most interesting of the substrates tested. At 5 min, trace amounts of glucose were evident. By 24 h all of the maltotriose was hydrolyzed to glucose and maltose. Figure 24 shows activity on maltose. Little activity was observed when maltose was the substrate. However, a trace amount of glucose was evident after 90 min of hydrolysis. No evidence of transglucosidase activity was observed on any of the chromatograms.

The enzyme was also characterized by using disc-gel electrophoresis. Because amylase in the pellet fraction was not eluted from DEAE-Sephadex A50 batch ion exchange chromatography, it was considered that the enzyme was attached to the exchanger by means other than ionic binding. To determine the type of proteins present, the proteins of the partially purified amylase were separated by disc-gel electrophoresis, then stained with preparations specific for proteins, glycoproteins and lipoproteins. Amylase activity was also assayed, the results are pictured in Figure 25. Proteins corresponding to amylase activity were located at the top of the gel. There was no evidence of any lipoprotein. Two bands of glycoprotein were visualized. The glycoprotein bands corresponded with a wide zone of amylase activity. A number of replications indicated that

Figure 23. Chromatographic analysis of Anaeroplasma bactoclasticum partially purified amylase action on maltotriose (reaction mixtures contained 5 mg/ml maltotriose and 0.1 ml/ml enzyme. The enzyme preparation produced amylase zone sizes of approximately 19.0 mm on the disc-plate assay)

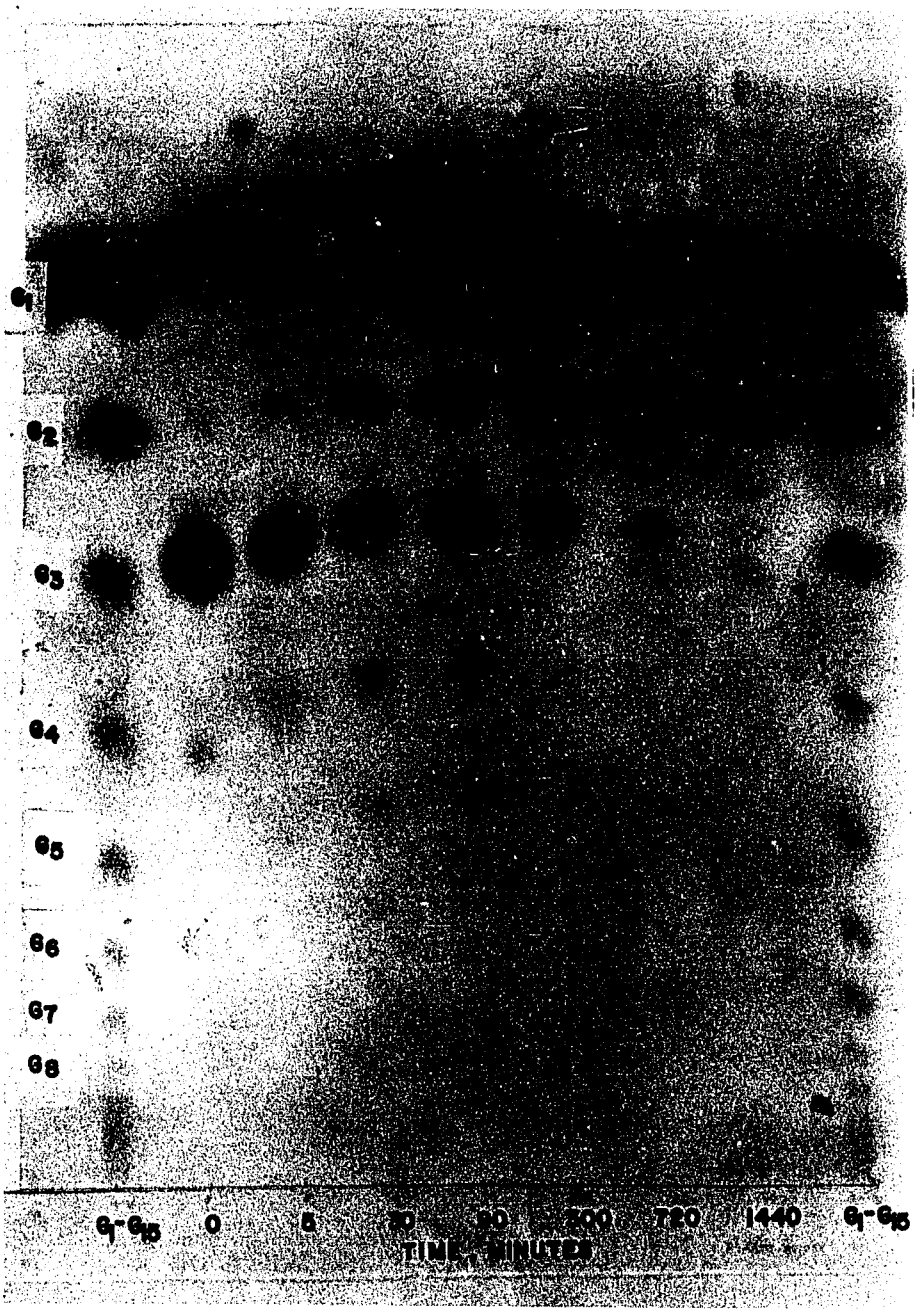

G1
G2
G3
G4
G5
G6
G7
G8
G1-G15
0
5
20
90
200
720
1440
G1-G15
TIME, MINUTES

Figure 24. Chromatographic analysis of Anaeroplasma bactoclasticum partially purified amylase action on maltose (reaction mixtures contained 5 mg/ml maltose and 0.1 ml/ml enzyme. The enzyme preparation produced amylase zone sizes of approximately 19.0 mm on the disc-plate assay)

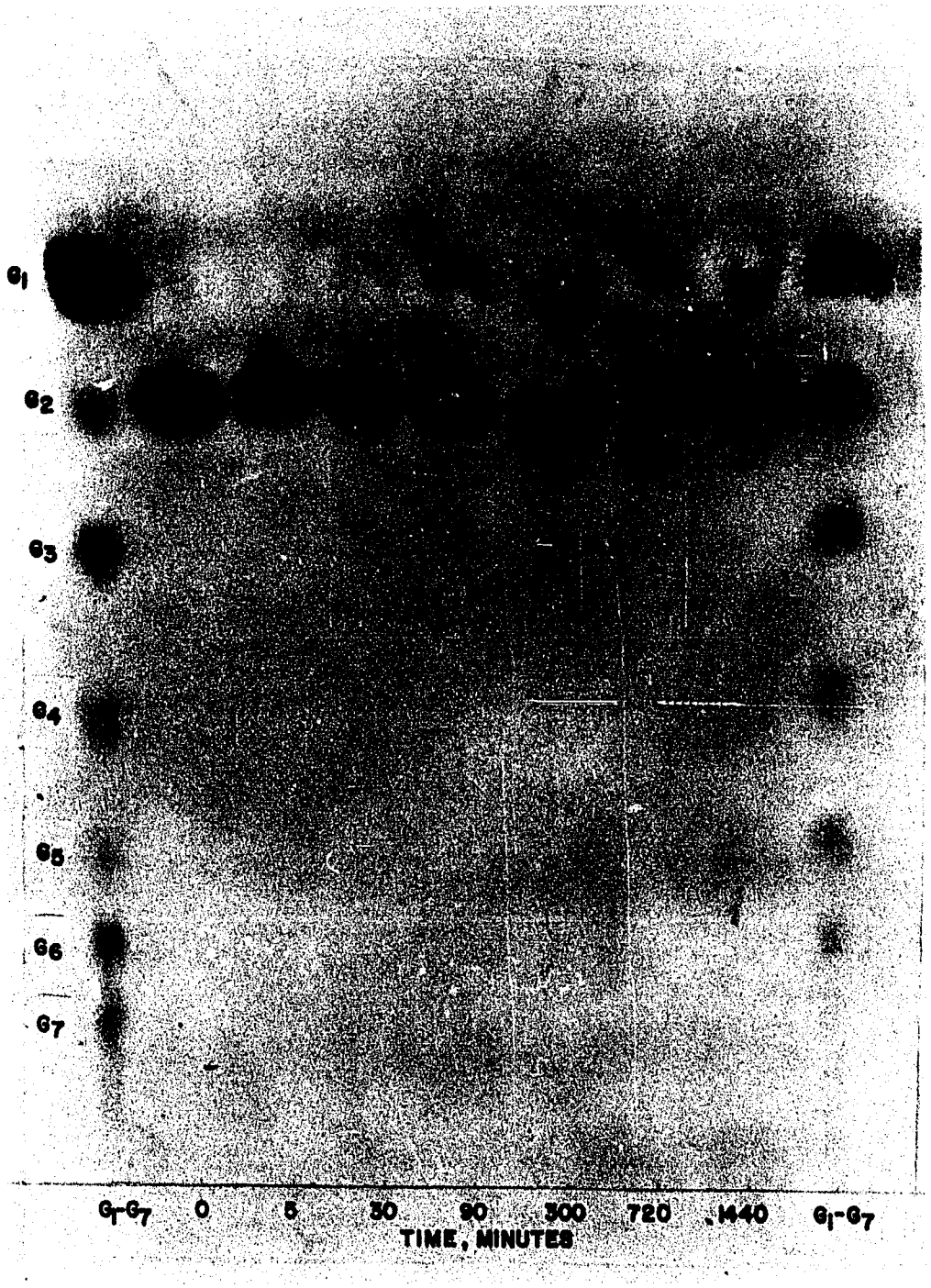
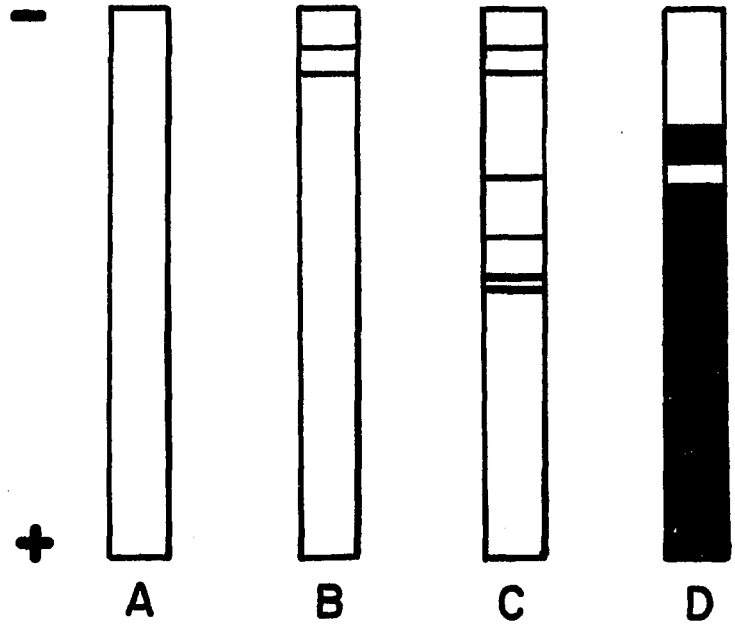

G1
G2
G3
G4
G5
G6
G7
G1-G7
0
5
30
90
300
720
1440
G1-G7
TIME, MINUTES

Figure 25. Disc-gel electrophoresis of the Anaeroplasma bactoclasticum partially purified amylase. (Gel A was stained for lipoproteins; Gel B for glycoproteins and Gel C for proteins. Gel D was used to detect amylase activity. After electrophoresis gels used for amylase detection were submerged in a soluble starch solution. After incubation the gels were flooded with an iodine solution. Clear zones indicated amylase activity)


-
+
A
B
C
D

6 bands of protein were present in the partially purified amylase preparation. Three protein bands possessed amylase activity and two of these were glycoproteins.

Ultrafiltration was used to determine the general molecular weight range of the partially purified amylase. Figure 26 shows that the fraction containing amylase activity had a molecular weight greater than 50,000 daltons. Sodium dodecyl sulfate disc-gel electrophoresis was used to determine the molecular weight of the enzyme preparation. The results are shown in Figure 27. The proteins in the partially purified amylase included a band of 125,000 daltons and a wide band, probably including several proteins, from 91,000 to 105,000 daltons.

Various ions have been found to effect the activity of microbial amylases. This topic has been discussed in the LITERATURE REVIEW. The effect of cations on Anaeroplasma bactoclasticum amylase was studied by using partially purified amylase that had been dialyzed against 0.01 M EDTA in 0.01 M PIPES at pH 6.0 for 24 h. The EDTA was removed by dialysis against 0.01 M PIPES, pH 6.0, for 24 h. The ions were incubated with the enzyme for 15 min at 42 C before being assayed. The results are shown in Table 11. The pH was verified before and after testing. Cations observed to significantly inhibit amylase activity included:

Figure 26. Separation of the amylase by molecular weight using ultrafiltration

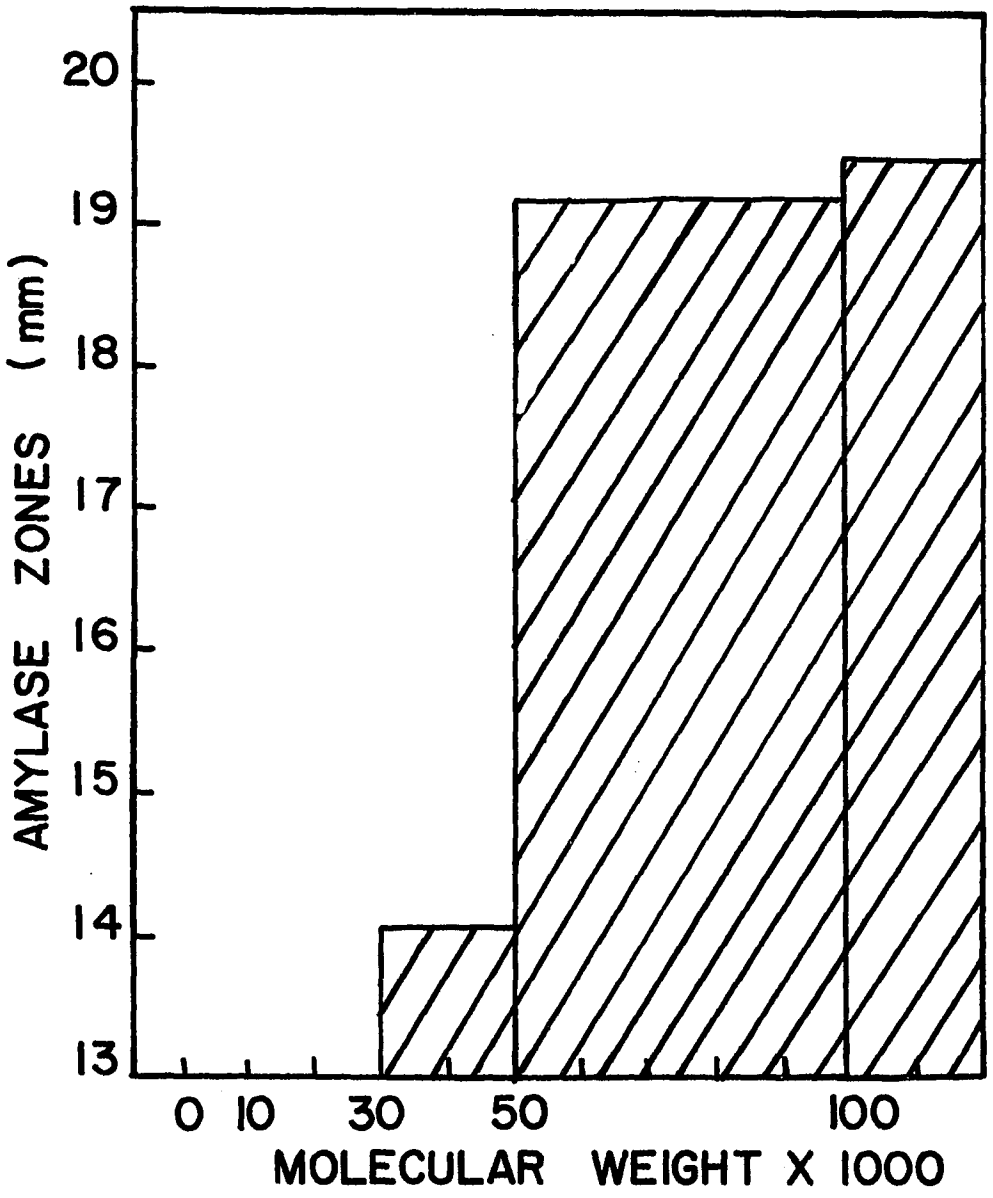

AMYLASE ZONES (mm)
20
19
18
17
16
15
14
13
0 10 30 50 100
MOLECULAR WEIGHT X 1000

Figure 27. Molecular weight determination of the Anaeroplasma bactoclasticum partially purified amylase by using SDS-polyacrylamide disc-gel electrophoresis (molecular weights of the standard proteins were: myoglobin, 17,200; ovalbumin, 43,000; γ-globulin (H-chain), 50,000; serum albumin, 68,000; and aldolase, 158,000)

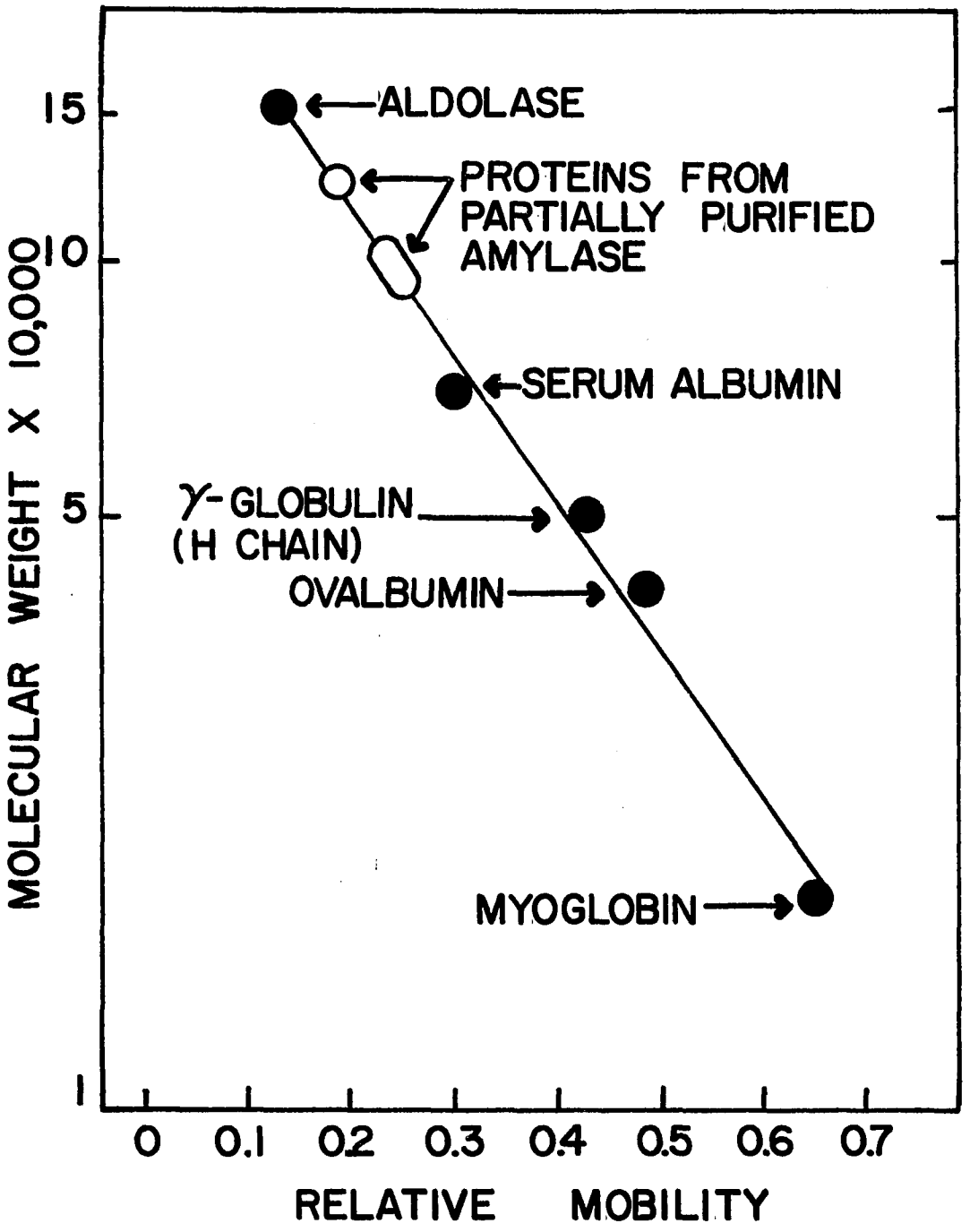

15
10
5
1
MOLECULAR WEIGHT X 10,000
ALDOLASE
PROTEINS FROM
PARTIALLY PURIFIED
AMYLASE
SERUM ALBUMIN
γ-GLOBULIN
(H CHAIN)
OVALBUMIN
MYOGLOBIN
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
RELATIVE MOBILITY

Table 11. Influence of cations and anions on Anaeroplasma bactoclasticum amylase activity[a]

Salt	Percentage Amylase Activity[b] Salt Concentration 5mM	1mM
LiCl	100	ND[c]
NaCl	96	96
$MgCl_2.6H_2O$	91	90
Na_2HPO_4	99	99
$AlCl_3$	100	100
$CaCl_2$	100	100
$FeCl_3$	63	63
$CoCl_2.6H_2O$	99	97
$NiCl_2.6H_2O$	97	96
$CuCl_2$	29	72
$ZnCl_2$	29	84
$SrCl_2.6H_2O$	99	99
$PdCl_2$	0	8
$AgNO_3$	0	32
$Cd.acetate.2H_2O$	33	50
$CsCl_2$	91	96
$BaCl_2.2H_2O$	96	96
$HgCl_2$	10	18
$Pb(NO_3)_2$	100	100

[a]0.01 M PIPES buffer pH 6.0 was used. The pH was verified before and after testing.

[b]When no cations were added to the EDTA-treated and dialyzed enzyme preparation, 100% amylase activity was observed.

[c]ND represents the experiment was not done.

Fe^{+3}, Cu^{+2}, Zn^{+2}, Pd^{+2}, Ag^{+2}, Cd^{+2}, and Hg^{+2}.

The influence of other potential effectors on amylase activity was also studied. The effectors were incubated with the enzyme at 42 C for 15 min prior to being assayed. These results are shown in Table 12. Effectors observed to significantly decrease amylase activity included: xylose, arabinose, δ-gluconolactone, mannose, α-methyl-D-mannoside, fructose, iso-maltotriose, EDTA, p-mercuribenzoate and N-bromosuccinimide.

Table 12. Influence of effectors on Anaeroplasma bactoclasticum amylase activity[a]

Effector	Percentage Amylase Activity Effector Concentration	
	10mM	5mM
xylose	85	85
arabinose	77	81
glucose	100	100
phenyl-α-D-glucoside	100	100
δ-gluconolactone	81	100
D-gluconate	100	100
D-glucosamine.HCl	100	100
galactose	100	100
galacturonic acid	100	100
mannose	88	90
α-methyl-D-mannoside	90	90
L-ascorbic acid	100	100
fructose	88	85
maltose	100	100
maltotriose	100	100
iso-maltotriose	ND[b]	91

[a] 0.01 M PIPES buffer, pH 6.0 was used. The pH was verified before and after testing.

[b] ND represents the experiment was not done.

Table 12 (Continued)

Effector	Percentage Amylase Activity Effector Concentration 10mM	5mM
maltotetraose	100	100
ethylene diamine tetraacetic acid	65	100
N-ethyl malimide	100	100
p-chloromercuribenzoate[c]	63	63
iodoacetimide	100	100
N-bromosuccinimide	63	88
dithiothreitol	100	100
L-cysteine	100	100

[a]Experiments with p-chloromercuribenzoate were performed at pH 8.0, whereas all other experiments were at pH 6.0.

DISCUSSION

The development of the optimal production medium for Anaeroplasma bactoclasticum 5LA extracellular amylase revealed interesting characteristics of this enzyme. As a general rule higher yields of microbial α-amylases are found in media containing complex starch materials than in defined media (Clary, Mitchell and Little, 1968; Coleman, 1967; Ensley, McHugh and Barton, 1975; Nyiri, 1971). Thus, it was not surprising that the highest levels of amylase production by this mycoplasma were in media containing maize starch. Other authors have assumed that the induction by starch is a result of hydrolytic products of starch because starch cannot penetrate the cell membrane (Ensley, McHugh and Barton, 1975). Consequently, low molecular weight hydrolytic products would be expected to stimulate amylase synthesis. This was the case for a number of microbial amylases. Bacillus stearothermophilus α-amylase synthesis has been induced by maltose, maltotriose, maltotetraose, maltopentaose and maltohexaose (Welker and Campbell, 1963b), while maltotetraose was observed to be the most effective inducer of B. licheniformis amylase (Saito and Yamamoto, 1975). Maltose increased production of Pseudomonas saccharophilia α-amylase (Markovitz and Klein, 1955).

The greatest induction of Anaeroplasma bactoclasticum amylase was observed when 0.75% maize starch was used as the carbon source. Paper chromatography verified that no detectable low molecular weight maltodextrins (glucose through maltoheptaose) contaminated the uninoculated maize starch. Therefore, the inducing factor was probably not maltodextrins contaminating the maize starch. However, maltodextrins produced in the early stages of growth may have caused induction. Amylase was produced when glucose, maltose and maltotriose were used as the sole carbon sources (Table 9). When compared to the amylase produced in media containing maize starch (100%), however, use of these oligosaccharides resulted in yields of only 67, 57 and 54%, respectively. Their addition to production medium containing maize starch did not increase amylase production (Table 9). Thus, it does not appear that low molecular weight maltodextrins are a major cause of the induction seen when maize starch was added to the medium.

It appeared that A. bactoclasticum synthesized amylase constitutively because the enzyme was produced in the absence of α1:4-glucosides. When cellobiose was the sole carbon source the level of mycoplasma amylase was 59% of that synthesized when maize starch was the carbon source (Table 9). Other microorganisms have been shown to synthesize amylase constitutively. These include ...

B. amyloliquifaciens (Coleman, 1967), B. subtilis (Sekiguchi and Okada, 1972) and B. licheniformis (Meers, 1972). B. polymyxa, however, only produced amylase when α-1,4-glucosidic materials were present in the growth medium (Fogarty and Griffin, 1973).

The theory that is now generally accepted is that low levels of constitutive enzymes are produced; starch is hydrolyzed by these constitutive enzymes and low molecular weight products that can enter the cell are produced, inducing further enzyme synthesis (Priest, 1977). If this hypothesis is true, it is probable that the maltodextrins with a larger degree of polymerization than tested in this study were responsible for the inducing effect of maize starch because significant induction was not apparent when glucose, maltose or maltotriose was added to the medium. However, it is possible that amylase induction could occur indirectly by attachment of the large molecular weight substrate at some membrane binding site. It is evident that the true mechanism of amylase induction by complex α-1,4-glucosidic materials warrants further investigation.

Anaeroplasma amylase production was greater in the presence of 40% rumen fluid than in media not containing rumen fluid. The addition of rumen fluid has been observed

to enhance growth of most rumen microorganisms (Grubb and Dehority, 1976). All of the components of rumen fluid are not known. A large degree of rumen fluid variation has been observed, even when the rumen fluid was collected on different days from one animal held under one set of conditions. Consequently, in this study it was necessary to verify that when rumen fluid was incorporated into the production medium, Anaeroplasma gave the same amylase production curve for each batch of rumen fluid. It is possible that sufficient substrates might have been present in the rumen fluid to cause the inducing effect. When rumen fluid was examined by paper chromatography, no maltodextrins (up to maltoheptaose) were detectable. It is interesting to note that when Blackburn (1968) added rumen fluid to a culture of Bacteroides amylophilus there was no effect on protease production.

The addition of 1% peptone also caused increased amylase production from Anaeroplasma. Peptone has been observed to stimulate amylase production in other microorganisms (Mahmoud, Taha and Attia, 1968). This effect was probably a result of peptone increasing the supply of amino acids necessary for amylase production.

Serum has been shown to enhance the growth of aerobic mycoplasmas by donating cholesterol from the low density

lipoprotein fraction (Slutzky et al., 1976; Slutzky et al., 1977a). The addition of serum to the production medium resulted in lower levels of both growth and amylase production by Anaeroplasma (Figure 5). Growth inhibition by the very low density lipoprotein fraction of serum has been observed for Acholeplasma laidlawii and Mycoplasma hominus (Slutzky et al., 1977b). It has been hypothesized that the toxic effect on these aerobic mycoplasmas was a result of their lipases releasing large quantities of fatty acids from this serum fraction. This has not been substantiated experimentally (Slutzky et al., 1977b). It is not known if this strain of Anaeroplasma produces lipase, only that serum adversely effected amylase production.

Tween-80 has been observed to increase secretion of extracellular cellulase (Reese and Maguire, 1969) and glucosyl-transferase (Umesaki, Kawai and Mutai, 1977). Reese and Maguire (1969) proposed several possible mechanisms for enhancement of enzyme yields by surfactants. Among these are the following hypothesis: (i) that the surfactant acts at the cell membrane effecting cell permeability, (ii) the surfactant protects the enzyme from inactivation, (iii) that because of increased permeability further induction by extracellular compounds was possible, and (iv) that an increased rate of secretion might result in increased

synthesis. Tween-80 did not increase the amount of amylase produced by Anaeroplasma (Table 5); in fact, the amount of amylase produced was reduced when Tween-80 was added. It is possible that the osmotic effect of the surfactant caused lysis of some of the mycoplasmas resulting in fewer organisms present to produce amylase. Growth was not monitored when Tween-80 was added.

The production of some microbial amylases is repressed by glucose (Glenn, 1976). Among those bacteria in which glucose represses amylase production are B. subtilis (Heineken and O'Connor, 1972; MacDonald-Green and Colarusso, 1964) and Vibrio parahaemolyticus (Tanaka and Iuchi, 1971). When glucose (0.5 or 0.75%) was added to the production medium of Anaeroplasma, the same amount of amylase was produced as in production medium alone. Consequently, glucose repression was not observed for this mycoplasma. It has been previously observed that using complex starches as carbon sources gave maximal induction and no catabolic repression (Priest, 1977).

Cyclic AMP is known to stimulate synthesis of a number of extracellular enzymes (Wang et al., 1979), including B. subtilis α-amylase (Saito and Yamamoto, 1975; Yu-wei et al., 1973). When filter-sterilized cyclic AMP was added to media for Anaeroplasma (Table 9) no significant effect on amylase production was observed.

Growth studies demonstrated that Anaeroplasma bactoclasticum 5LA followed a typical bacterial growth curve with a generation time of 4 h and 20 min in the amylase production medium (Figure 6). This is not unusual because most mycoplasmas have a generation time ranging from 1 to 6 h (Maniloff and Morowitz, 1972). Anaeroplasma readily adapted to increasing the culture volume to 9 l. This was advantageous because increasing culture volume for large scale enzyme production is a problem with other enzyme-producing microorganisms. Another interesting observation, that was not experimentally investigated, was that serial transfer of this organism appeared to increase the amount of amylase produced. Consequently, after the preliminary studies each culture was transferred three times before inoculating test media. Each culture was grown 24 h before transfer.

Amylase production paralleled the growth curve (Figure 6 and 7). This is not the case with all amylases; B. subtilis does not initiate amylase synthesis until the stationary growth phase is reached (Coleman, 1967; Nomura, Maruo and Akabori, 1956; Priest, 1977). Proteins formed parallel with growth are primary metabolites, while secondary metabolites are generally formed after growth (Coleman, Brown and Stormonth, 1975). Therefore, under this definition,

Anaeroplasma amylase was a primary metabolite.

This extracellular amylase was easily separated from the bulk of the other proteins in the cell-free extract by ammonium sulfate precipitation; however, further purification was extremely difficult.

Disc-gel electrophoresis revealed six bands of protein, with three of these bands possessing amylase activity. Further purification by DEAE-Sephadex A50, DEAE-Sepharose CL-6B or Biogel A 0.5 m was unsuccessful because little or no protein separation was observed. When Sephadex G-200 was used (Figure 10) some separation was observed as compared to Biogel A 0.5 m (Figure 11). Sephadex is a glucose polymer. Thus, it is possible that the amylase might have bound to sephadex, allowing the amylase to elute slightly behind the other proteins. A sample from the eluate of the Sephadex-G200 (Figure 10, the fraction from 60 to 300 ml) was concentrated by ultrafiltration and then analyzed by disc-gel electrophoresis. Results indicated that the nonamylase protein of the partially purified preparation that was closest to the positive pole in Figure 26 was no longer present.

This binding to Sephadex-G200 may help to explain the reaction of this amylase with DEAE-Sephadex A50. When this ion exchanger was used for ion exchange column chromatography, only a trace amount of amylase was eluted. An analysis of

ion exchangers (Table 10) showed that 100% of the amylase was absorbed to the exchanger and only 23% was eluted with 1 M NaCl. When this absorption and elution pattern was compared with other DEAE-exchangers (Table 10) it was evident that the tight binding of this amylase to DEAE-Sephadex A50 was more involved than binding to DEAE. It is possible that the combination of enzyme binding to the sephadex and the DEAE-functional groups formed a strong bond that could not be broken by increasing the NaCl molarity of the buffer. Elution of the enzyme from DEAE-Sepharose CL-6B (Figure 12) implies that this enzyme will elute from nonglucose polymer DEAE-ion exchangers.

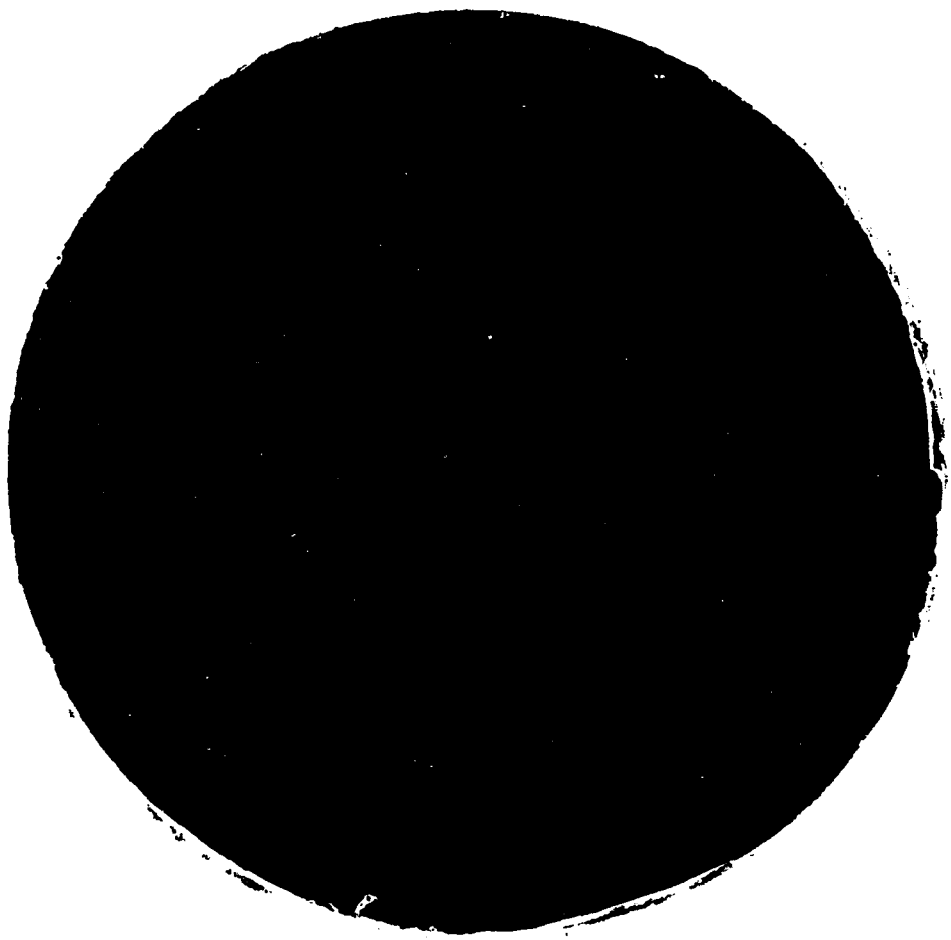
Molecular weight analyses (Figure 27) gave more information why purification was difficult. The partially purified amylase contained a protein of approximately 125,000 daltons and proteins ranging from 91,000 to 105,000 in size. Consequently, separation by molecular weight was not successful.

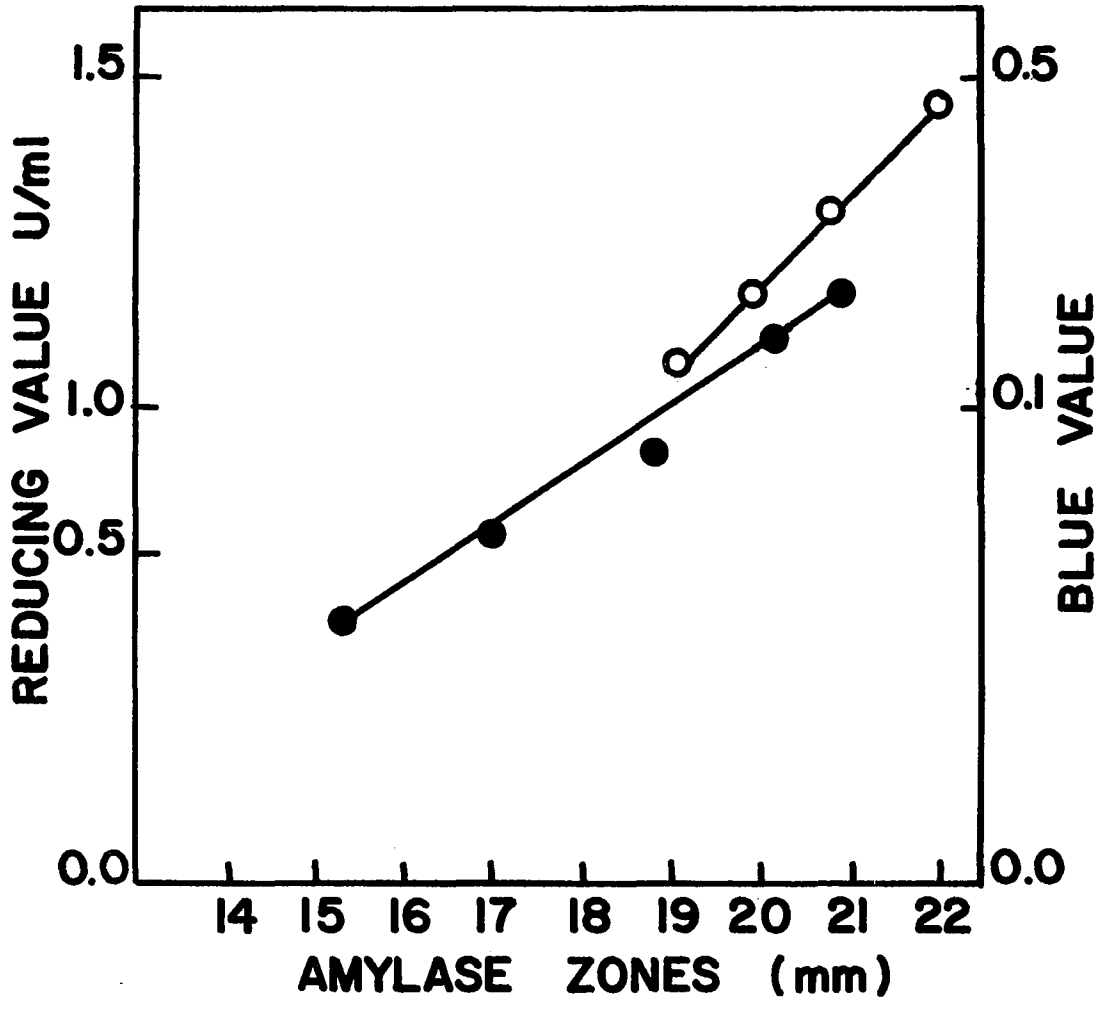
Throughout this study three enzyme assays were used to monitor enzyme activity: the reducing value, the blue value and the disc-plate assay. Boyer (1969) found that there was a linear relationship between the $\log_{10}$ of the reducing value and the disc-plate assay zone diameter for Streptococcus bovis α-amylase; a zone diameter increase of 1 mm correlated

with a log increase in reducing value. Thus, a 1 mm increase in zone size would represent a 10-fold increase in amylase activity.

Results comparing Anaeroplasma amylase by these three assays were different from those observed for S. bovis. In Figure 28 the $\log_{10}$ of the reducing and blue values were plotted against amylase zone diameter for Anaeroplasma bactoclasticum 5LA extracellular amylase. It is evident that a large amount of hydrolysis was necessary before a reduction in blue value was observed. It was also noted that a 1 log cycle increase of reducing value correlated with a 4.5 mm increase in zone diameter, while a 1 log cycle increase in the blue value corresponded with a 1 to 1.5 mm zone diameter increases.

As mentioned earlier, problems with the assays were first noticed when assaying amylase precipitated by ammonium sulfate. When the reducing value assay was performed virtually the same results were obtained from comparable samples containing the active enzyme as enzyme denatured by boiling 20 min. This indicated that the reducing activity observed was not a property of the amylase, but rather was caused by unknown reducing factors in the production medium. There was also no correlation between the reducing value and the disc-plate assay data for the ammonium




1.5
1.0
0.5
0.0
REDUCING VALUE U/ml
0.5
0.1
0.0
BLUE VALUE
14 15 16 17 18 19 20 21 22
AMYLASE ZONES (mm)

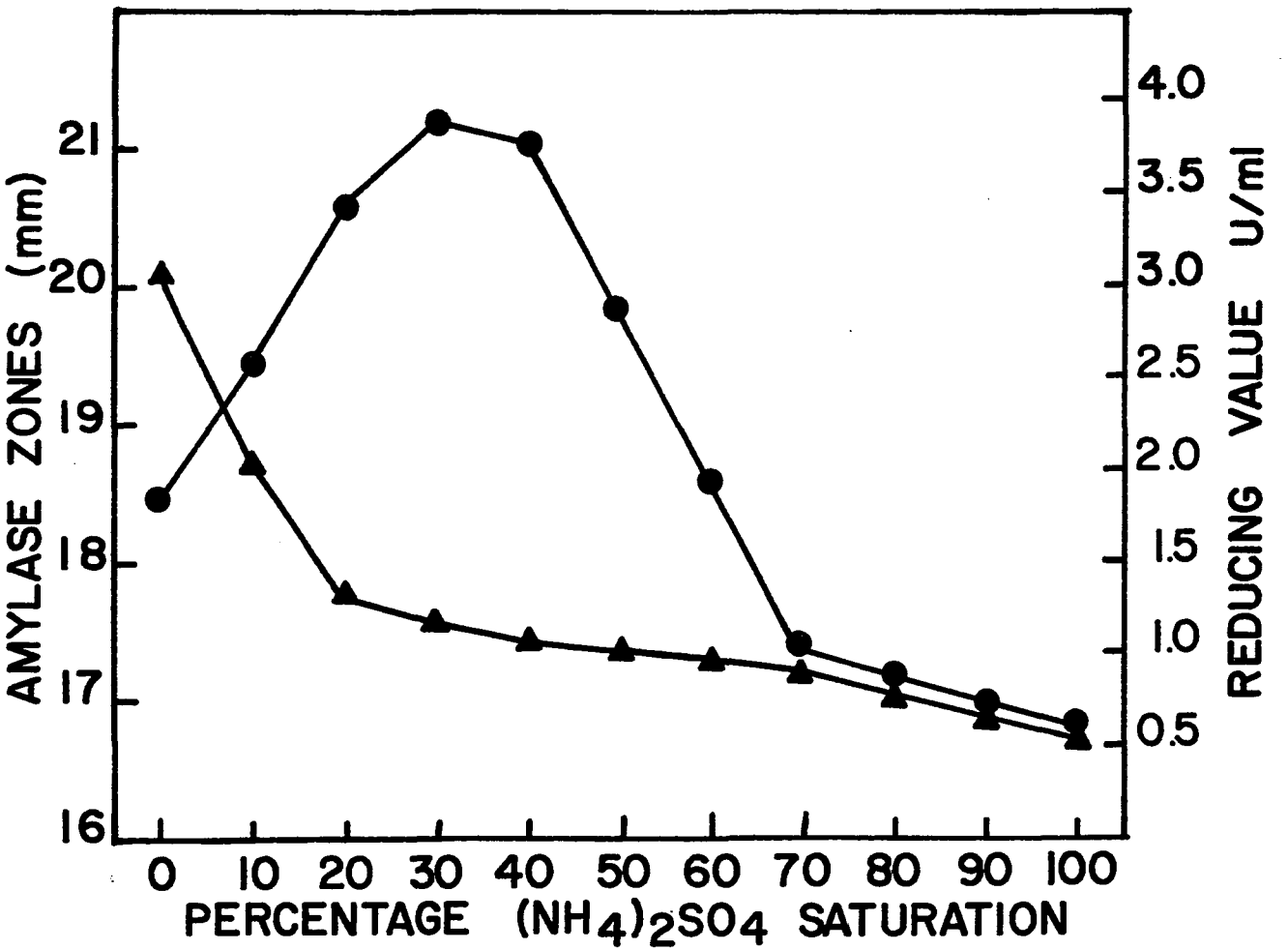
sulfate saturation curve, Figure 29. The disc-plate assay confirmed that there was no amylase activity following 20 min of boiling. Blue value data in the ammonium sulfate saturation experiments were erratic.

To examine possible interference by medium constituents on enzyme activity measurements, several medium components were added to the partially purified amylase preparation at the concentrations found in the production medium. The following media components were tested: clarified rumen fluid, peptone, production medium and spent production medium. The latter was tested to detect the effect of Anaeroplasma end-products on the amylase assays. Controls included a spent production medium control to monitor any increases resulting from amylase present in the spent medium.

Interference of the reducing value assay was evident. When peptone was added to the enzyme preparation the same results were obtained as with the control. All of the other components tested gave reducing values two to four times that of the control. This was not surprising because clarified rumen fluid contains several reducing agents.

The control gave a very low blue value (0.03), but the disc-plate assay showed an average zone diameter of 18.3.

Figure 29. Amylase activity in the supernatant fluid after the stepwise addition of ammonium sulfate ((●——●) symbolizes data obtained using the disc-plate amylase assay, while (▲——▲) symbolizes data obtained by the reducing value assay. These data are averages of three experiments)


AMYLASE ZONES (mm)
21
20
19
18
17
16
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
REDUCING VALUE U/ml
0 10 20 30 40 50 60 70 80 90 100
PERCENTAGE (NH4)2SO4 SATURATION

This indicated that the blue value results were not reflecting the true activity of this enzyme. When supplemented with medium components, larger blue values were detected. Blue values obtained for some medium components were: clarified rumen fluid, 0.19; peptone, 0.16; clarified rumen fluid plus peptone, 0.15; production medium, 0.13; and spent production medium, 0.19. This indicated that blue value data obtained prior to separating the enzyme from medium components was probably inaccurate.

Medium components also interferred with detection using the disc-plate assay. The control was an average zone size of 18.3 mm. When clarified rumen fluid was added there was no effect on the assay. When production medium, spent production medium, peptone, or clarified rumen fluid and peptone were added to the enzyme preparation, however, the average zone size increased to about 19.0 mm. Because all of these contained peptone, it was believed that the peptone was responsible for the increase in zone diameter. Checking to see if total protein concentration influenced the zone size, 1.0% bovine serum albumin (Sigma Chemical Co., St. Louis, MO) was added to Bacillus subtilis α-amylase. The increase of total protein did not effect amylase zone size in this instance.

Consequently, the accuracy of all three assays was

questioned. The disc-plate assay was considered the most reliable and was used throughout the characterization study. Because of the unrealiability of the assays, specific activity and percentage yield calculations made throughout the purification process were also concluded to be inaccurate and are, therefore, not included in this dissertation.

The pH and temperature optima (Table 1) were comparable to those observed for other amylases. The amylase was resistant to temperature denaturation at temperatures normally found in the rumen (Figure 16). It was sensitive to elevated temperatures, however, because no activity was observed after incubation at 62 C for 60 min.

Paper chromatography (Figure 23) revealed that this amylase was most active on maltotriose, hydrolytic products were detectable after 5 min of incubation. Less activity was observed on larger substrates. This pattern is unlike the amylase of B. subtilis which acts more slowly on short dextrins than on amylose (Greenwood and Milne, 1968). The conversion of substrates to glucose and maltose as final products was apparent. The high degree of activity on maltotriose appeared to be significant because other microbial amylases are known to have low activity on maltotriose (Bird and Hopkins, 1954), whereas glucoamylases would result in complete hydrolysis to glucose (Fleming, 1968). It is significant that other amylases of microorganisms

from the bovine and ovine rumen have been observed to have no action on maltotriose; these include Streptococcus bovis and Clostridium butyricum (Hobson and MacPherson, 1952). Also, bovine and ovine pancreatic amylases produce glucose only in small amounts (Clary, Mitchell and Little, 1968). Thus, the production of glucose and maltose from accumulated maltotriose in the rumen may be a significant role of Anaeroplasma bactoclasticum in the rumen.

The production of large amounts of glucose by this enzyme was reminiscent of glucoamylase activity. However, glucoamylases are primarily described as exoenzymes, producing only glucose (Fleming, 1968). Anaeroplasma amylase was only weakly active on maltose (Figure 24). The molecular weight of most glucoamylases is approximately 100,000 daltons (Fleming, 1968). One component(s) of the Anaeroplasma amylase preparation was similar to the glucoamylases, with a molecular weight near 100,000 daltons (Figure 27), rather than the 50,000 daltons that is common to most microbial amylases (Table 1).

Two of the three proteins with amylase activity appeared to be glycoproteins (Figure 25). Others have studied enzymes that are glycoproteins (Glenn, 1976); however, this characteristic appears to be unique among microbial α-amylases.

As discussed in the LITERATURE REVIEW, the effect of ions on microbial amylase activity has been studied extensively by many authors. Removal of cofactors by incubation with 10 mM EDTA (Table 12) was inhibitory to amylase activity. However, in the ion experiment (Table 11), analysis against 10 mM EDTA was not inhibitory to amylase activity since amylase zone sizes were similar whether or not $CaCl_2$ was added to the EDTA-treated enzyme preparation. These data indicated that Ca^{+2} was not completely removed by dialysis against EDTA. The inhibitory effect of $FeCl_3$ was interesting because Coleman and Elliott (1962) observed that $FeCl_3$ increased B. subtilis amylase production. No effect on Anaeroplasma amylase production was observed. When the partially purified amylase was incubated with $FeCl_3$, the activity was decreased by 37%. As discussed in the LITERATURE REVIEW, a number of other authors have observed inhibitory effects of mercury, silver and copper on amylases. These heavy metals also inhibited the activity of Anaeroplasma amylase (Table 11). Amylase from this mycoplasma was not inhibited by lead. This is contrary to the inhibition observed for other microbial amylases (DiCarlo and Redfern, 1947; Greenwood and Milne, 1968; Urata, 1957).

The influence of effectors on amylase activity showed that the five carbon sugars, xylose and arabinose, were

inhibitory to amylase activity. This was probably a result of competitive inhibition. Generally, the six carbon sugars did not effect the enzyme activity with the exception of mannose, fructose, and δ-gluconolactone. Larger oligosaccharides did not effect activity.

It is highly probable that the reason lead, maltotetraose (a possible competitive inhibitor) and other effectors were not inhibitory was a function of the disc-plate assay. The concentrations listed in Tables 11 and 12 were those applied to the paper-discs. Following application of the saturated discs to the assay agar, the enzyme and effector would begin to diffuse through the agar. Unbound effectors would probably diffuse through the agar faster than the larger protein. Thus, the concentration of the effector acting on the enzyme would be close to zero. Consequently, only the results of these experiments showing inhibition indicate the influence of the effectors, and conclusions from chemicals not causing inhibition must be viewed with caution.

This reasoning may also apply to the lack of protease detection when 0.1% dithiothreitol was added to the cell-free extract before assaying by a protease disc-plate assay. Consequently, these data do not disprove the hypothesis that a protease inhibited by oxidation may be

present.

This theory may explain the contradictory results concerning phosphate inhibition. Experiments determining optimum conditions for the disc-plate assay indicated that phosphate in the disc-plate assay agar was somewhat inhibitory to this amylase; however, it was later observed (Table 11) that Na_2HPO_4 was not inhibitory to amylase activity. It is possible that the phosphate on the discs diffused away from the enzyme.

The reducing agents, dithiothreitol and L-cysteine, did not effect amylase activity; the oxidizing agent, N-bromosuccinimide, inhibited activity. The sulfhydral group modifiers, N-ethyl malimide and iodoacetimide did not effect amylase activity; however, activity was inhibited by p-chloromercuribenzoate. N-bromosuccinimide was also inhibitory to the α-amylases of Bacterioides amylophilus (McWethy, 1975), and B. subtilis (Onoue, Okada and Yamamura, 1962). Consequently, because amylase assays were performed in an aerobic atmosphere which may have oxidized the enzyme to some degree, it is possible that greater amylase activity is present in the natural anaerobic habitat of the enzyme.

The inaccuracy of the amylase assays used throughout this study was a serious problem. For further study of this

and other amylases produced by rumen microorganisms, the development of an accurate amylase assay (one that is not affected by components of rumen fluid) is essential. Because oxidation can effect some enzymes activity, the development of an assay that can be performed under obligate anaerobic conditions would be desirable. An accurate assay is necessary in order for specific activity and percent yield calculations to be made. Also, an assay must be developed before kinetic experiments can be performed.

Because of its anaerobic nature Anaeroplasma bactoclasticum is unique among the mycoplasmas. This study originated because it was believed that this unusual organism may produce an enzyme with unique properties. The amylase of Anaeroplasma bactoclasticum has been shown to have properties similar to other microbial amylases, but there are also some unique properties.

Unlike other microbial amylases purification by ion exchange and Sephadex G-200 chromatography was unsuccessful. These problems were probably associated with properties of the contaminating proteins. It is possible that the ammonium sulfate precipitation step of purification may be altering the enzyme affecting purification by ion exchange chromatography. Consequently, further efforts

at amylase purification need to be undertaken.

The hydrolytic action pattern of this enzyme is a distinguishing factor. As previously discussed,the enzyme has a high affinity for maltotriose and produces large amounts of maltose and glucose from a number of carbon sources. This function may make a significant contribution to the utilization of carbohydrates in the rumen. The similarities of the enzyme's action and molecular weight with glucoamylases may also be significant. Furthermore, the unique property of producing glucose and maltose as final products from large molecular weight carbohydrates may have industrial applications.

SUMMARY

An ovine rumen strain of the obligately anaerobic mycoplasma, Anaeroplasma bactoclasticum produces an extracellular amylase. This organism, unique among the mycoplasma because of its anaerobic nature, was selected because it might produce amylase with unusual properties.

An extensive series of experiments on media formulations for amylase production resulted in the development of an optimal amylase production medium. As observed with other amylase-producing microorganisms, cultures of Anaeroplasma grown in media containing complex α-1,4-glucosides produced the highest levels of amylase. Levels of amylase production were also significantly increased when clarified rumen fluid and Bacto-peptone were added to the culture medium. The optimal amylase production medium contained: clarified rumen fluid (40% w/v), Bacto-peptone (1% w/v), maize starch (0.75% w/v), cholesterol (0.002% w/v), resazurin (0.0001% w/v), cysteine.HCl (0.05% w/v), Na_2CO_3 (0.4% w/v)/ penicillin G (1000 U/ml) and the mineral solution of Bryant and Burkey (1953) with the $CaCl_2$ concentration increased to 0.005% w/v.

Unlike some other amylases, the Anaeroplasma enzyme was produced throughout the entire growth phase. Amylase production was not subject to glucose repression. Also, the

addition of cyclic AMP did not effect amylase production. Apparently, low levels of this amylase were produced constitutively because amylase was detected when the organism was grown in media not containing added α1,4-glucosides.

The extracellular amylase was easily separated from the bulk of the other proteins in the cell-free extract by ammonium sulfate precipitation; however, further purification could not be affected when techniques that had been used to purity amylases from other sources were used. No further protein separation was attained by using column chromatography with DEAE-Sephadex A50, DEAE-Sepharose CL-6B, or Biogel A 0.5 m. Very little separation was observed when Sephadex G-200 was used. Consequently, a partially purified enzyme preparation which had been precipitated by ammonium sulfate and decolorized by DEAE-Sephadex A50 batch ion exchange was used to obtain the general characteristics of the amylolytic enzyme(s) produced by A. bactoclasticum.

Throughout this study, three amylase assays were used. These were the disc-plate assay (a modification of Stark et al., 1953), and reducing value and blue value assays (Robyt and Whelan, 1968). Constituents of the production medium interfered with amylase activity measurements

in all three assays. Problems with the enzyme assays prevented accurate determinations of specific activity and percent yield. The disc-plate assay, modified to provide optimum conditions for Anaeroplasma, was the most accurate means of detecting amylase activity.

The optimum pH of the partially purified amylase was 6.0; the temperature optimum was 42-45 C. The crude enzyme preparation contained six proteins, as determined by disc-gel electrophoresis. Three of the protein bands possessed amylase activity; two of the bands possessing amylase activity were glycoproteins. One protein of the partially purified amylase had a molecular weight of 125,000 daltons; another, diffuse band, appeared in the range from 91,000 to 105,000 daltons.

Anions observed to inhibit amylase activity included: Fe^{+3}, Cu^{+2}, Zn^{+2}, Pd^{+2}, Ag^{+2}, Cd^{+2}, and Hg^{+2}. Effectors that inhibited amylase activity were: xylose, arabinose, δ-gluconolactone, mannose, α-methyl-D-mannoside, fructose, iso-maltose, ethylene-diamine tetraacetic acid, p-chloromercuribenzoate, and N-bromosuccinimide.

One of the most interesting characteristics observed was the amylase action pattern. Unlike some other microbial amylases, the major hydrolytic products produced by this amylase were maltose and glucose. The enzyme had a high degree of activity on maltotriose, a carbohydrate that is

not hydrolyzed by other known microbial amylases of the rumen. Consequently, the hydrolysis of maltotriose and production of large quantities of glucose may be a significant role of this organism in the rumen.

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